



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>G01N 33/53, C07K 5/02, 1/04</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/35199</b> <b>(43) International Publication Date:</b> 25 September 1997 (25.09.97)
<b>(21) International Application Number:</b> PCT/US97/04963 <b>(22) International Filing Date:</b> 20 March 1997 (20.03.97) <b>(30) Priority Data:</b> 60/013,822      20 March 1996 (20.03.96)      US <b>(60) Parent Application or Grant</b> (63) Related by Continuation US      60/013,822 (CIP) Filed on      20 March 1996 (20.03.96) <b>(71) Applicant (for all designated States except US):</b> THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10550 North Torrey Pines Road, La Jolla, CA 92037 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> JANDA, Kim, D. [US/US]; 3181 Eric Street, San Diego, CA 92117 (US). HAN, Hyunsoo [KR/US]; 7190 Shoreline Drive #6101, San Diego, CA 92122 (US). <b>(74) Agents:</b> LEWIS, Donald, G. et al.; The Scripps Research Institute, 10550 North Torrey Pines Road, TPC-8, La Jolla, CA 92037 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> AZATIDE PEPTIDOMIMETICS		
<b>(57) Abstract</b>		
<p>Peptidomimetic azatides and combinatorial oligoazotide libraries are produced by means of a stepwise synthesis. Combinatorial library construction of this new biomimetic polymer provides a means to fabricate global peptidomimetic libraries.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

- 1 -

## AZATIDE PEPTIDOMIMETICS

DescriptionTechnical Field:

5 The present invention relates to compounds that  
mimic peptides. More particularly, the present  
invention relates to the synthesis of peptides in which  
 $\alpha$ -carbons of the peptide backbone have been replaced by  
trivalent nitrogen atoms using either solution phase or  
liquid phase synthetic methodologies.

10

Background:

Peptidomimetics have become immensely important  
for both organic and medicinal chemists (Spatola et al.  
*Chemistry and Biochemistry of Amino Acids, Peptides,  
and Proteins*; Weinstein, B., Ed.; Marcel Dekker: New  
York, 1983; pp. 267-357; Sherman et al. *J. Am. Chem.  
Soc.* **1990**, *112*, 433; Hirschmann et al. *Angew. Chem.  
Int. Ed. Engl.* **1990**, *29*, 1278; Gante et al. *Angew.  
Chem. Int. Ed. Engl.* **1994**, *33*, 1699). Synthetic  
20 interest in these surrogate peptide structures has been  
driven by the pharmaceutical industry's needs for  
molecules with improved pharmacokinetic properties  
(Hodgson et al. *Bio/Technology* **1993**, *11*, 683).  
Biophysical studies on these pseudopeptides has allowed  
25 elucidation of the functional role of the peptide  
backbone (Marshall et al. *Chemical Recognition in  
Biological Systems*; Creighton et al. The Chemical  
Society: London, 1982; p 278; Farmer et al. *Drug  
Design*; Ariens, E. J., Ed.; Academic Press, New York,  
30 1980, p. 121) and with an ever-increasing level of  
synthetic sophistication the degree of peptide mimicry  
within a peptidomimetic can be tailored to chemist's  
needs. Indeed, the alteration of peptides to  
peptidomimetics has included peptide side chain

- 2 -

manipulations, amino acid extensions (Freidinger et al. *Science* 1980, 210, 656; Paruszewski et al. *Rocz. Chem.* 1973, 47, 735; Stachowiak et al. *J. Med. Chem.* 1979, 22, 1128), deletions (Rivier et al. *Chemia* 1972, 26, 303; Sarantakis et al. *Clin. Endocrinol.* 1976, 5, 2755), substitutions, and most recently backbone modifications (Hagihara et al. *J. Am. Chem. Soc.* 1992, 114, 6568; Simon et al. *Proc. Natl. Acad. Sci. USA* 1992, 89, 9367; Smith et al. *J. Am. Chem. Soc.* 1992, 114, 10672; Cho et al. *Science* 1993, 261, 1303; Liskamp et al. *Angew. Chem. Int. Ed. Engl.* 1994, 33, 633; Burgess et al. *Angew. Chem. Int. Ed. Engl.* 1995, 34, 907). It is this latter development that has been exploited for the synthesis of biomimetic polymeric structures. Such progress has been fueled by the suggestion that peptidomimetics may provide novel scaffolds for the generation of macromolecules with new properties of both biological and chemical interest.

The most common manipulation involving the  $\alpha$ -carbon atom of peptides is the inversion of stereochemistry to yield D-amino acids (Spatola et al. *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*; Weinstein, B., Ed.; Marcel Dekker: New York, 1983; pp. 267-357). The importance of this substitution in affording compounds with improved biological potencies, altered conformational properties (Mosberg et al. *Proc. Natl. Acad. Sci. USA* 1983, 80, 5871), and increased resistance to enzymatic degradation has been widely recognized and exploited (Dooley et al. *Science* 1994, 266, 2019). Replacement of the  $\alpha$ -hydrogen of the common amino acids by a methyl group, or by any other substituents ( $\text{NH}_2\text{CRR}'\text{CO}_2\text{H}$ ) are both further examples of  $\alpha$ -alkyl modification. Azapeptides, however, are peptides in which one (or more) of the  $\alpha$ -carbon(s) has been replaced by a

- 3 -

trivalent nitrogen atom (Figure 5) (Gante et al. *Synthesis* 1989, 405). This transformation results in a loss of asymmetry associated with the  $\alpha$ -carbon and yields a structure that can be considered intermediate in configuration between D- and L- amino acids (Aubry et al. *Biopolymers* 1989, 28, 109). Interest in this  $\alpha$ -carbon replacement unit stems from its ability to provide resistance to enzymatic cleavage and its capacity to act as a selective inhibitor of cysteine (Magrath et al. *J. Med. Chem.* 1992, 35, 4279) and serine proteases (Elmore et al. *Biochem. J.* 1968, 107, 103; Barker et al. *Biochem. J.* 1974, 139, 555; Gray et al. *Tetrahedron* 1977, 33, 837; Gupton et al. *J. Biol. Chem.* 1984, 259, 4279; Powers et al. *J. Biol. Chem.* 1984, 259, 4288). While the synthesis of azapeptides has been reported (Bentley et al. *J. Chem. Soc. (C)* 1966, 60; Dutta et al. *J. Chem. Soc. Perkin Trans 1* 1975, 1712; Furr et al. *J. Chem. Soc. Perkin Trans 1* 1979, 379; Quibell et al. *J. Chem. Soc. Perkin Trans 1* 1993, 2843), the synthesis of a "pure azapeptide", or what we will term an "azatide" has yet to be accomplished. The earliest attempts to make pure azatides can be dated to Gante and co-workers. (Gante et al. *Chem. Ber.* 1965, 98, 3340; Gante et al. *Proc. Am. Pept. Symp.* 13th. 1993, 1994, 299) However, the methodology that was reported does not allow azatide stepwise chain lengthening in a repetitive manner of anything but hydrazine units.

The utility of azatide compounds has been demonstrated in the treatment of various disorders including cancers, viral infections and cataracts. For example, Moretti et al, *J. Clin. Endocrino. Metab.*, 81(11), 3930-3937, 1996, show that azatide compounds are used as LH-releasing hormone agonists to interfere with stimulatory actions of epidermal growth factor in

- 4 -

human prostatic cancer cell lines. Jeyarajah et al, *Gynecol. Oncol.*, 63(1), 47-52, 1996, use an azatide gonadotropin-releasing hormone analog for treatment of recurrent endometrial cancers. Brower et al, *J. Surg. Res.*, 52(1), 6-14, 1992, show differential effects of azatide containing LHRH and somatostatin analogs on human breast cancers. Hellberg et al., PCT Int. Appl. WO 9640107 A1 961219, demonstrate the use of (N,N'-bis(mercaptoacetyl) hydrazine derivatives as anticataract agents. Nakashima et al., EP 672678 A1 950920, show the preparation and use of azapeptide compounds as neurokinin A antagonists. Azatide type compounds have been used as retroviral protease inhibitors, Kempf et al., PCT Int. Appl. WO 9414436 A1 940707, lipoxxygenase inhibitors (Atkinson et al Eur. Pat. Appl. EP 146243 A1 850626) and immunosuppressant rapamycin carbamate analogs, Kao et al. US Appl. US 5411967A 950502.

What is needed is either a solution phase or liquid phase synthetic methodology for synthesizing azatides using monomeric " $\alpha$ -aza-amino acids" which can be coupled in a linear and stepwise chain-lengthening fashion. What are needed are azatides as mimetics for peptides which are easy to synthesize, more stable and more active than the parent peptides. Moreover, azatide mimetics are needed for stability as compared to various natural peptide products and compounds which possess better bioavailability and exhibit greater activity as compared to known peptides.

- 5 -

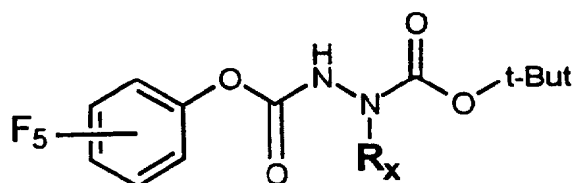
Summary of the Invention:

The invention is directed to the azatides and a method for the synthesis of azatide mimetics. In particular, an efficient method has been developed for the solution and liquid phase syntheses of biopolymer mimetics consisting of " $\alpha$ -aza-amino acids" linked in a repetitive manner to form an azatide oligomer. A general synthetic procedure is claimed which provides the use and synthesis of a wide variety of Boc-protected aza-amino acid monomers with optimization of solution phase procedures for the coupling of aza-amino acids in a repetitive manner. In addition, the design and synthesis of a linker is employed that supports azatide synthesis using a liquid phase synthetic format. Oligoazatides can now be rapidly assembled on a homogeneous polymeric support. Using the methodology provides a potential source of new peptidomimetic libraries.

One aspect of the invention is directed to a process for synthesizing an oligoazatide. The process employs a support material with a linker unit attached thereto. The preferred support material is a soluble homopolymer support, e.g., polyethylene glycol monomethyl ether (MeO-PEG). Polyethylene glycol monomethyl ether is soluble in aqueous media but precipitates in ether. Precipitation of the support material with ether can be employed for purifying coupled molecules. Alternative soluble supports having this property are conventional and may be readily substituted for the polyethylene glycol monomethyl ether. Solid phase supports may also be employed but are less preferred because of their poorer yields and/or difficulty in handling.

- 6 -

A linker unit is attached to the soluble support. A preferred linker unit is *p*-hydroxymethylbenzoate. The process also employs a Boc-protected aza-amino acid. Preferred  
5 Boc-protected aza-amino acids are represented by the following structure:



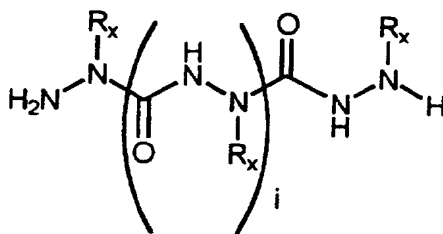
wherein  $R_x$  is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl,  $C_1$ - $C_6$  alkyl, benzyl, substituted benzyl and the side chain radical  
10 of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val. The Boc-protected aza-amino acid is reacted with a carbonyl activation  
15 element for producing an activated carbamate of the Boc-protected aza-amino acid. A preferred carbonyl activation element is bis-pentafluorophenyl carbonate. The soluble homopolymer support is then coupled with the activated carbamate for producing a nascent  
20 protected chain. The nascent protected chain may then be deprotected using a mild acid for producing a nascent deprotected chain. A preferred mild acid for the deprotection step is trifluoroacetic acid. The nascent deprotected chain is then washed by  
25 precipitation of the soluble homopolymer. The nascent deprotected chain may then be extended by repeating the above "n" times wherein  $1 \leq n \leq 100$  and wherein the soluble homopolymer support of said Step A is replaced with the nascent deprotected chain for producing an

- 7 -

extended deprotected chain. Then, the extended deprotected chain is decoupled from the soluble support by hydrogenation for producing the oligoazatide.

5 Another aspect of the invention is directed to a process for producing a combinatorial oligoazatide library. The process employs "n" reaction vessels wherein nascent chains are extended by the addition  
10 respectively of the activated carbamates of "n" Boc-protected aza-amino acids. After each extension step, aliquots of the products are saved and cataloged and the remainder is pooled into a common pot to form a mixture. The mixture of nascent chains is then  
15 aliquoted into "n" reaction vessels for further extension. After "m" extensions, the product is decoupled and separated from the soluble support to form the combinatorial oligoazatide library.

20 Another aspect of the invention is directed to a combinatorial oligoazatide library. The library comprising a plurality of compounds represented by the following formula:



25

wherein  $0 \leq i \leq 99$  and  $\text{R}_x$  is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl,  $\text{C}_1$ -

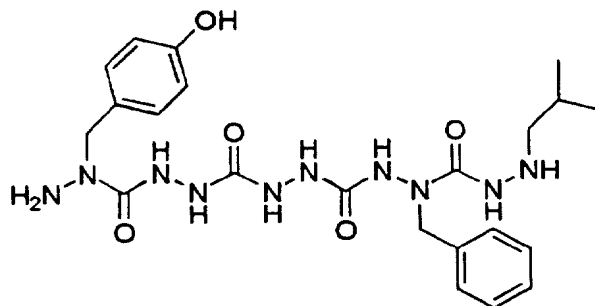
- 8 -

C<sub>6</sub> alkyl, benzyl, substituted benzyl and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

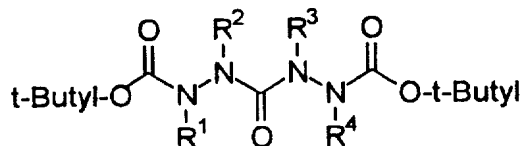
5

Another aspect of the invention is directed to an azatide compounds represented by the following formulas:

10



and



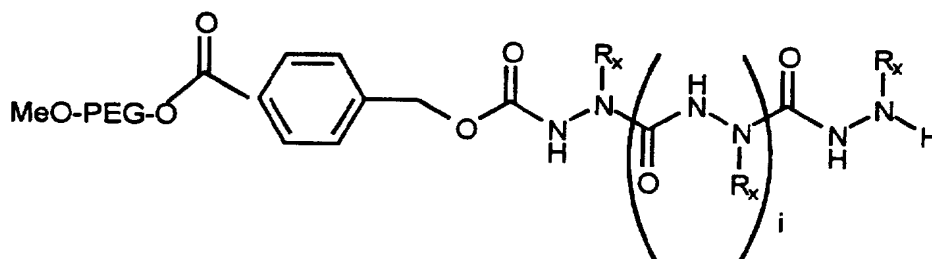
15

wherein R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> and R<sub>4</sub> is selected from the group consisting of hydrogen, methyl, isopropyl, isobutyl and benzyl.

Another aspect of the invention is directed to

- 9 -

intermediate oligoazatide compounds represented by the following formula:



- 5 wherein  $0 \leq i \leq 99$  and  $R_x$  is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl,  $C_1$ - $C_6$  alkyl, benzyl, substituted benzyl and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, 10 Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

#### Description of Figures:

- 15 Figure 1 shows a table which indicates yields of the preparation of various diazatides starting from 1-R-hydrazine carboxylic acid, 1,1-dimethylethyl ester wherein R ( $R^1$ ,  $R^2$ ,  $R^3$  and  $R^4$ ) is represented by the indicated functional groups.

- 20 Figure 2 illustrates the preparation of Boc-protected alkyldiazine monomers wherein R,  $R^1$  and  $R^2$  are selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl, benzyl, and the side chain radical of the following amino acids: Ala, Arg, Asn, 25 Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

- 10 -

Figure 3 illustrates various routes for solution phase diazatide synthesis wherein  $R^1$  and  $R^2$  are selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl, benzyl, and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

Figure 4 illustrates the synthesis of a MeO-PEG-supported Leu-enkephalin azatide mimetic.

Figure 5 illustrates the comparison of a peptide, azapeptide, and azatide wherein  $R^1$ ,  $R^2$  and  $R^3$  are selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl, benzyl, and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

Figure 6 illustrates the fragmentation patterns of  $(M + 1)^+$  ion of peptides and azatides wherein  $R^1$  and  $R^2$  are selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl, benzyl, and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

Figure 7 illustrates CAD spectra of  $m/z$  517  $(M + 1)^+$  and 403 peaks for compound 16.

Figure 8 illustrates a combinatorial strategy for the synthesis of a library of azetide molecules, starting from a support-linker molecule and coupling an 'addition molecule' to such support-linker molecule in a separate reaction vessel, saving, cataloging, and subsequently recombining each product to form a library

- 11 -

of compounds with a nascent protected chain of length 1 wherein  $R_x$  is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl, benzyl, and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val; other functionalities are possible.

Figure 9 illustrates a combinatorial strategy for the synthesis of a library of azetide molecules, starting from a nascent deprotected chain of length 1 (compound 18) and coupling an 'addition molecule' to such support-linker molecule in a separate reaction vessel, saving, cataloging, and subsequently recombining each product to form a library of compounds with a nascent protected chain of length 2 wherein  $R_x$  is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl, benzyl, and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val; other functionalities are possible.

Figure 10 illustrates a combinatorial strategy for the synthesis of a library of azetide molecules, starting from a nascent deprotected chain of length 2 (compound 20) and coupling an 'addition molecule' to such support-linker molecule in a separate reaction vessel, saving, cataloging, and subsequently recombining each product and repeating "i" iterations ("i" is an arbitrary number depending on the size of the azetide desired eg. from 0 to 100 cycles) of the cycle of steps 1 and 2 to form a library of azetide products wherein  $R_x$  is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl, benzyl, and the side chain radical of the following amino acids: Ala,

- 12 -

Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val; other functionalities are possible.

5           Figure 11 illustrates the synthesis of activated pentafluorophenyl carbamate-azetides for the common amino acids.

10           Figure 12 illustrates the synthesis of activated pentafluorophenyl carbamate-azetides for the common amino acids.

15           Figure 13 illustrates the synthesis of activated pentafluorophenyl carbamate-azetides for the common amino acids.

20           Figure 14 illustrates the synthesis of activated pentafluorophenyl carbamate-azetides for the common amino acids.

          Figure 15 illustrates activated pentafluorophenyl carbamate-azetides for the common amino acids.

25           Figure 16 illustrates activated pentafluorophenyl carbamate-azetides for the common amino acids.

#### Detailed Description of the Invention

30           The invention is directed to azatides and the design and general synthesis of oligoazatide mimetics. Thus, for the synthesis of oligoazatides, an alphabet of suitably protected aza-amino acid constituents are needed. The invention embodies a methodology to synthesize *de novo* Boc-protected alkylhydrazine  
35           monomers substituted with a variety of functional groups. Two principal routes are used in their

- 13 -

syntheses (Figure 2): (1) Reduction of Boc-protected hydrazones (Dutta et al. *J. Chem. Soc. Perkin Trans 1* 1975, 1712), derived from the reaction of Boc-carbazate with either an aldehyde or ketone (Figure 2; equation 1). (2) Alkylation of hydrazine with an alkylhalide, followed by Boc-protection of the resulting alkylhydrazine (Figure 2; equation 2) (Biel et al. *J. Am. Chem. Soc.* 1959, 81, 2805). The outgrowth of these methods is the transient protection of either the "amino or carboxy-terminal" functionality of the aza-amino acid and an ability to create a unique alphabet of  $\alpha$ -aza-amino acid R-groups.

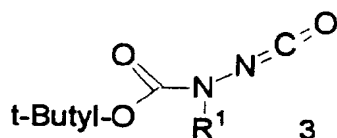
To convert these Boc-protected aza-amino acids into acylating agents that would allow stepwise chain lengthening, the hydrazine portion of the molecule had to be activated (Figure 3). Activation of this moiety is a challenging problem since the Boc-alkylhydrazines are poorer nucleophiles than simple amines or amino acids. Consequently, we required a highly activated carbonyl synthon that would allow facile coupling of two Boc-protected aza-amino acids to form the azatide-linkage. Furthermore, this coupling reaction had to be controllable, such that symmetrical dimer formation could be minimized. Our initial attempts to couple two aza-amino acids together using *p*-nitrophenyl chloroformate, carbonyldiimidazole, bis-(2,4-dinitrophenyl) carbonate, or trichloromethyl chloroformate were unsuccessful, as they suffered from complicated side-reactions, poor reaction yields, and/or prolonged reaction time. We reasoned that these results were due to either the insufficient leaving ability of *p*-nitrophenol and imidazole, or steric hindrance of the *o*-nitro group in the case of bis-(2,4-dinitrophenyl) carbonate. To overcome these problems we opted to use bis-pentafluorophenyl

- 14 -

carbonate 1 as the carbonyl activation element (Efimov  
et al. *Nucleic Acids Research* 1993, 21, 5337). Our  
decision to use this reagent was based on three  
factors. First, the pentafluorophenol functionality is  
5 a powerful electron-withdrawing group, while the fluoro  
substituents minimize steric problems. Second, the  
bis-pentafluorophenyl carbonate can be readily prepared  
from phosgene and a sodium pentafluorophenolate  
solution. Third, the compound is a highly crystalline  
10 solid which is easy to handle.

Shown in (Figure 3) are two solution phase routes  
to diazatides. In the first case, carbamate 2 is  
utilized for the coupling reaction. Thus, a Boc-  
15 protected aza-amino acid is added dropwise to 1  
granting activation of the 1-R' hydrazinecarboxylic  
acid, 1,1-dimethylethyl ester. The activated complex  
formed, 2, is not isolated but instead immediately  
reacted via the addition of a second Boc-protected  
20 alkylhydrazine to complete the diazatide coupling.  
This coupling procedure provides diazatides in good  
yield with few side reactions in an acceptable reaction  
time. Results using this coupling method are summarized  
in (Figure 1). From this table, it is evident that the  
25 coupling process is quite general, as both simple Gly<sup>a</sup>-  
Gly<sup>a</sup> (superscript a refers to an aza-amino acid linkage)  
and sterically demanding (Val<sup>a</sup>-Val<sup>a</sup>) azatides can be  
synthesized in less than an hour. The latter result is  
extremely important as it dictates whether the stepwise  
30 coupling of aza-amino acids is feasible. Whereas  
coupling through activated 1-R' hydrazinecarboxylic  
acid, 1,1-dimethylethyl ester was successful, the  
coupling of activated 2-R' hydrazinecarboxylic acid,  
1,1-dimethylethyl ester was not (Figure 3). From these  
35 findings, we surmise that the activated complex is not  
carbamate 2, but rather the isocyanate 3 as follows:

- 15 -



Findings reported by Abeles (Magrath et al. *J. Med. Chem.* 1992, 35, 4279) support this disclosure. For an activated 2-R' hydrazinecarboxylic acid, 1,1-dimethylethyl ester, the intermediate is untenable because of the carbamate's substitution pattern.

The techniques described above allow  $\alpha$ -azatide chain building to be performed in an iterative manner. To prepare a small well-defined  $\alpha$ -azatide, we chose to use polymer-supported liquid phase synthesis (Geckeler et al. *Advances in Polymer Science*; Abe, A. et al. Ed.; Springer-Verlag: Berlin, 1995, Vol. 121, p. 31). Liquid phase synthesis uses a soluble linear homopolymer [polyethylene glycol monomethyl ether (MeO-PEG)] which serves as a terminal protecting group for the compound to be synthesized. The essence of this technology is that it avoids a number of difficulties found in solid-phase synthesis and preserves the positive aspects of solution phase synthesis. We have demonstrated the advantages of using liquid phase synthesis through the construction of both peptide and small molecule combinatorial libraries (Han et al. *Proc. Natl. Acad. Sci. USA* 1995, 92, 6419).

A leucine-enkephalin peptide sequence, (YGGFL), was chosen as the first azatide mimetic to be synthesized. This pentamer was selected as the N-terminal sequence within this unit, (YGGF), is common to most natural opioid peptides (Meo et al. *Proc. Natl.*

- 16 -

*Acad. Sci. USA* 1983, 80, 4084). The successful diazotide coupling procedure described in (Figure 3) implies N-to-C-terminal construction of the azatide. A *p*-substituted benzyl ester spacer unit that would  
5 accommodate directional synthesis on MeO-PEG and withstand the rigors of Boc-chemistry was designed (12, Figure 4). It was reasoned that 12 attached to MeO-PEG would be stable against acidolysis due to the presence of the *para*-benzoate substituent, and the oligoazatide  
10 could be liberated by catalytic hydrogenation generating a free amino group. Thus methyl *p*-(hydroxymethyl) benzoate was *O*-protected as the *t*-butyl ether by treatment with isobutylene and acid. Subsequent hydrolysis of the methyl ester with lithium  
15 hydroxide provided 12. Linker 12 was coupled to MeO-PEG with the aid of DCC/DMAP and upon deprotection with trifluoroacetic acid (TFA) gave the MeO-PEG-benzyl-OH (13) support ready for azatide synthesis. Synthesis of the azatide pentamer Y<sup>a</sup>G<sup>a</sup>G<sup>a</sup>F<sup>a</sup>L<sup>a</sup> was accomplished in a  
20 repetitive stepwise fashion as shown in (Figure 4). Because of the unique physical properties of the MeO-PEG homopolymer each coupling/deprotection reaction could be purified by precipitation of the modified homopolymer. Furthermore MeO-PEG allows reaction  
25 progress to be conveniently monitored by either proton-NMR spectroscopy or the Kaiser ninhydrin test (Kaiser et al. *Anal. Biochem.* 1979, 34, 595). Based on our linker strategy, the pentamer and the benzyl protecting group of aza-tyrosine could be liberated in a single  
30 step using catalytic hydrogenation to give the Boc-protected pentamer (overall yield: 56.7 % from 13). This compound was converted to the desired Leu-enkephalin azatide by treatment with trifluoroacetic acid (Figure 4).

35

Tandem mass spectrometry (Hunt et al. *Proc. Natl.*

- 17 -

*Acad. Sci. USA.* **1986**, *83*, 6233; Biemann et al. *Methods in Enzymology* **1990**, *193*, 455), when coupled with any soft ionization method, has emerged as an important tool for the elucidation of sequences of peptides and nucleotides. We used this technique for the sequence determination of our azatide. Thus, the Leu-enkephalin azatide was subjected to ESI-tandem mass spectrum analysis. In the acidic matrix employed for ESI experiments, Leu-enkephalin azatide would exist as a  $(M+H)^+$  ion with a proton located on the  $\alpha$ -nitrogen atom of Leu<sup>a</sup> (i.e. the most basic residue). In the gas phase,  $(M+H)^+$  ions undergo proton-transfer to other basic sites to allow charge delocalization. For an azatide, proton transfer would preferentially occur on the more basic tertiary amide nitrogens over the secondary amide nitrogens. The protonation of a tertiary amide nitrogen causes bond-cleavage between the  $\alpha$ -nitrogen and carbonyl carbon to generate X- and A-type fragments (Figure 6). When there is no preferential protonation between two secondary amide nitrogens such as in the urea-linkage involving the Gly<sup>a</sup> residues, cleavage is possible on either side of the carbonyl group. Conversely, Y- and B-type fragmentations of peptides results from protonation of amide nitrogens and hence cleavage of amide bonds (Figure 6). This prediction was manifested in the collision-induced dissociation (CAD) pattern of Leu-enkephalin azatide **16** shown in (Figure 7). The MS-MS of the  $(M+H)^+$  ion at 517 produced daughter peaks at 403, 255, 197 (A-type), 321, 263 (X-type) and MS-MS-MS on 403 ( $M\text{-Leu}^a\text{+H}^+$ ) gave grand-daughter peaks at 255, 197, 139 (A-type), 207, 149 (X-type), 239, 197, 123 (Y-type). Peaks at 297, 149, 107, 91 represent A-type fragments involving cleavage of side-chain of Tyr<sup>a</sup>. Mass difference between homologous A-type ions corresponds to elements -CONHNR-. Predicted  $m/z$  values for  $A_2\text{-}A_5$

- 18 -

fragments were obtained by sequentially adding the incremental masses of Gly<sup>a</sup>, Gly<sup>a</sup>, Phe<sup>a</sup>, and Leu<sup>a</sup> to that for A<sub>1</sub> at 139. A similar argument can be made for X-type and Y-type fragments, confirming the Tyr<sup>a</sup>-Gly<sup>a</sup>-Gly<sup>a</sup>-Phe<sup>a</sup>-Leu<sup>a</sup> sequence of Leu-enkephalin azatide.

The azatide oligomer sequence synthesized, (Tyr<sup>a</sup>-Gly<sup>a</sup>-Gly<sup>a</sup>-Phe<sup>a</sup>-Leu<sup>a</sup>), provided a chance to assess any biological activity that this azatide biopolymer sequence may possess (*vide supra*). Monoclonal antibody 3-E7 was raised by Meo et al. against the antigen  $\beta$ -endorphin and, like the  $\delta$ -opioid receptor, recognizes the N-terminal portion of the protein (Meo et al. *Proc. Natl. Acad. Sci. USA* 1983, 80, 4084). The antibody also binds tightly to [Leu<sup>5</sup>]-enkephalin [Tyr-Gly-Gly-Phe-Leu], ( $K_d = 7.1$  nM) and a variety of related opioid peptides (Cwirla et al. *Proc. Natl. Acad. Sci. USA* 1990, 87, 6378). A competition ELISA method was used to investigate if the Tyr<sup>a</sup>-Gly<sup>a</sup>-Gly<sup>a</sup>-Phe<sup>a</sup>-Leu<sup>a</sup> sequence could bind to IgG 3-E7 (Han et al. *Proc. Natl. Acad. Sci. USA* 1995, 92, 6419). At 1mM the azatide pentamer showed no propensity to compete with the natural peptide for 3-E7. While this result at first glance appears to be disappointing it was not completely unexpected. The bound conformation(s) of enkephalin have been studied extensively for over the past 15 years (Garner et al. *Tetrahedron* 1993, 49, 3433). While the exact bioactive conformation of this peptide remains shrouded, it is thought that the active form of this peptide resides in some sort of a  $\beta$ -turn (Bradbury et al. *Nature* 1976, 260, 165; Lowe et al. *Proc. Natl. Acad. Sci. USA* 1978, 75, 7; Manavalan et al. *Int. J. Pept. Protein Res.* 1981, 18, 256). This being based on x-ray crystallographic data which showed that the glycine residues at the second and third position of enkephalin force a type I'  $4 \rightarrow 1$   $\beta$ -turn (Smith et al.

- 19 -

Science 1978, 199,, 1214; Ishida et al. *Biochem. J.* 1984, 218, 677). Although we have yet to obtain an x-ray structure on 16, physiochemical data does exist on diacyl hydrazines (Olivato et al. *J. Chem. Soc., Perkin Trans. II* 1983, 1053; Graybill et al. *Bioorg. & Medicinal Chem. Lett.* 1992, 2, 1375). Simple unsubstituted diacyl hydrazine's (i.e. glycine azatides) contain a dihedral ( $\phi$ ) angle of approximately -175 degrees. While N-substituted ( i.e. all other azatides) possess a dihedral ( $\phi$ ) angle of approximately -110 degrees. Taken as a whole this data suggests that 16 should adopt a more extended conformation within the critical glycine region. In essence then this azatide oligomer would have difficulty in achieving the orientation displayed by the antigenic determinant (Tyr-Gly-Gly-Phe-Leu) that elicited IgG 3-E7. The outcome being that 16 is non-ligand for 3-E7.

- 20 -

**SYNTHETIC PROTOCOLS****General**

<sup>1</sup>H and <sup>13</sup>C nmr spectra were recorded either on a  
5 Bruker AM-250, a Bruker AMX-400 or a Bruker AMX-500  
spectrometer. Residual protic solvent CHCl<sub>3</sub> ( $\delta_H = 7.26$   
ppm,  $\delta_C = 77.0$ ), d<sub>4</sub>-methanol ( $\delta_H = 3.30$  ppm,  $\delta_C = 49.0$ )  
and D<sub>2</sub>O ( $\delta_H = 4.80$  ppm,  $\delta_C$  (of CH<sub>3</sub>CN) = 1.7 ppm) or TMS  
( $\delta_H = 0.00$  ppm) were used as internal reference.  
10 Coupling constants were measured in Hertz (Hz). HRMS  
were recorded using FAB method in a m-  
nitrobenzylalcohol (NBA) matrix doped with NaI or CsI.  
Infra-red spectra were recorded on a Perkin-Elmer FTIR  
1620 spectrometer. Enantiomeric excess was determined  
15 by HPLC using a Daicel Chemical Industries CHIRALPAK AD  
column. Optical rotations were measured with an Optical  
Activity AA-1000 polarimeter. Melting points were taken  
on a Thomas Hoover capillary melting point apparatus  
and are uncorrected. Column chromatography was  
20 performed on Merck Kieselgel 60 (230-400 mesh).  
Analytical thin layer chromatography was performed  
using pre-coated glass-backed plates (Merck Kieselgel  
F<sub>254</sub>) and visualized by cerium molybdophosphate or  
ninhydrin. Diethyl ether, tetrahydrofuran (THF) and  
25 toluene (PhCH<sub>3</sub>) were distilled from sodium-benzophenone  
ketyl, dichloromethane (DCM) and acetonitrile from  
calcium hydride. Other solvents and reagents were  
purified by standard procedures if necessary. The  
optical rotations were measured with a JASCO DIP-1000KUY  
30 automatic digital polarimeter. <sup>1</sup>H and <sup>13</sup>C-NMR spectra  
were measured with a JEOL EX 270 and/or a 500 FT-NMR  
spectrometer and chemical shifts were given on a  $\delta$   
(ppm) scale with tetramethylsilane as an internal  
standard. The FAB-MS were measured with a JEOL DX-300  
35 and/or SX102A spectrometer. MALDI-TOF Mass was  
measured under the condition: Positive mode, 6kV,

- 21 -

Reflectron with gentisc acid as the matrix by Kratos  
Kompact (Shimazu, MALDI-III). TLC was performed on  
precoated Kieselgel 60 F<sub>254</sub> plates (Merck). Column  
chromatography was carried out on Kieselgel 60 (70-230  
5 mesh and 230-400 mesh) and MCI gel CHP-20P (Mitsubishi  
Chemical, Ind.). Polyethylene glycol monomethyl ether  
(MeO-PEG, MW. = 5000) was purchased from Aldrich and  
was dried over P<sub>2</sub>O<sub>5</sub> under vacuum before use. Boc-  
protected methylhydrazines, isopropylhydrazines,  
10 isobutylhydrazines, benzylhydrazines, *p*-(*O*-  
benzyl)hydroxybenzylhydrazines, and pentafluorophenyl  
carbonate were prepared according to literature  
procedures (DMAP is 4-*N*-dimethylaminopyridine).

15 **General Solution Phase Diazatide Coupling Procedure as  
illustrated in Figure 3, equation 1:**

To a stirred solution of pentafluorophenyl  
carbonate 1 (50.0 mg, 13.0 mmol; synthesis *vida infra*)  
in methylene chloride (5 ml) over a period of 20 min, a  
20 solution of 1-(*N*-Boc)-alkylhydrazine (1 eq; synthesis  
*vida infra*) and DMAP (1 eq) in methylene chloride (2  
ml) is added dropwise to form compound 2. Upon  
completion of the addition, a solution of 2-(*N*-Boc)-  
alkylhydrazine (1 eq; synthesis *vida infra*) and DMAP (1  
25 eq) in methylene chloride (2 ml) are added. The  
resulting mixture was stirred for 30 min at room  
temperature. Removal of solvent and flash  
chromatography (Still et al. *J. Org. Chem.* 1978, 43,  
2923) gives the desired unsymmetrical diazatide.

30 **Boc-Gly<sup>2</sup>-Gly<sup>2</sup>-Boc Case:**

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>) δ 1.35 (s, 18H), 7.33  
(broad s, 2H), 7.77 (s, 2H); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>) δ  
27.9, 81.3, 156.2, 157.1; HRMS (FAB) calcd for [C<sub>11</sub>H<sub>22</sub>N<sub>4</sub>O<sub>5</sub>  
35 + Cs<sup>+</sup>] 423.0645, found 423.0655.

- 22 -

**Boc-Ala<sup>2</sup>-Ala<sup>2</sup>-Boc Case (symmetrical):**

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>) δ 1.42 (s, 18H), 3.09 (s, 6H), 7.43 (s, 2H); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>) δ 28.1, 38.3, 81.5, 156.1, 157.0; HRMS (FAB) calcd for [C<sub>13</sub>H<sub>26</sub>N<sub>4</sub>O<sub>5</sub> + Cs<sup>+</sup>] 451.0958, found 451.0976.

**Boc-Ala<sup>2</sup>-Ala<sup>2</sup>-Boc Case:**

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>) δ 1.43 (s, 9H), 1.45 (s, 9H), 3.08 (s, 3H), 3.09 (s, 3H), 6.45 (broad s, 1H), 7.05 & 7.62 (broad s, 1H); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>) δ 28.2, 28.3, 37.9, 81.2, 81.4, 155.4, 156.6, 156.8; HRMS (FAB) calcd for [C<sub>11</sub>H<sub>22</sub>N<sub>4</sub>O<sub>5</sub> + Cs<sup>+</sup>] 451.0958, found 451.0965.

**Boc-Ala<sup>2</sup>-Phe<sup>2</sup>-Boc:**

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>) δ 1.37 (s, 9H), 1.41 (s, 9H), 3.1 (s, 3H), 4.50 (broad s, 2H), 6.10 & 6.59 (broad s, 1H), 7.22 (m, 5H), 7.37 & 7.55 (broad s, 1H); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>) δ 27.8, 28.2, 38.5, 54.3, 81.5, 81.6, 127.3, 128.3, 128.6, 137.3, 155.5, 156.4, 156.5; HRMS (FAB) calcd for [C<sub>19</sub>H<sub>30</sub>N<sub>4</sub>O<sub>5</sub> + Cs<sup>+</sup>] 527.1271, found 527.1289.

**Boc-Ala<sup>2</sup>-Leu<sup>2</sup>-Boc:**

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>) δ 0.90 (d, J = 7 Hz, 6H), 1.40 (s, 9H), 1.45 (s, 9H), 1.84 (m, 1H), 3.07 (s, 3H), 3.35 (broad s, 2H), 6.30 & 6.56 (broad s, 1H), 7.20 & 7.36 (broad s, 1H); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>) δ 19.9, 26.3, 28.1, 28.3, 38.0, 55.7, 81.1, 82.0, 154.5, 156.1, 157.6; HRMS (FAB) calcd for [C<sub>16</sub>H<sub>32</sub>N<sub>4</sub>O<sub>5</sub> + Cs<sup>+</sup>] 493.1427, found 493.1447.

**Boc-Leu<sup>2</sup>-Leu<sup>2</sup>-Boc:**

<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>) δ 0.88 (d, J = 7 Hz, 6H), 0.90 (d, J = 7 Hz, 6H), 1.42 (s, 9H), 1.46 (s, 9H), 1.86 (m, 2H), 3.27 (broad s, 4H), 6.33 & 6.57 (broad s,

- 23 -

1H), 7.11 & 7.23 (broad s, 1H); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>) δ 20.4, 20.4, 27.6, 27.8, 28.5, 28.6, 56.7, 59.5, 81.8, 82.3, 156.3, 156.5, 158.1; HRMS (FAB) calcd for [C<sub>19</sub>H<sub>38</sub>N<sub>4</sub>O<sub>5</sub> + Cs<sup>+</sup>] 535.1897, found 535.1881.

5

**Boc-Val<sup>2</sup>-Val<sup>2</sup>-Boc:**

Melting point was 101-102°C; <sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>) δ 1.09 (broad s, 12H), 1.40 (s, 9H), 1.45 (s, 9H), 4.32 (broad s, 1H), 4.61 (m, 2H), 6.27 (broad s, 1H), 6.79 (broad s, 1H); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>) δ 19.3, 19.8, 28.0, 28.3, 48.4, 48.6, 81.0, 81.6, 157.1, 157.4, 157.9; HRMS (FAB) calcd for [C<sub>17</sub>H<sub>34</sub>N<sub>4</sub>O<sub>5</sub> + Cs<sup>+</sup>] 507.1584, found 507.1599.

10

15

**Linker Preparation:**

**Methyl *p*-(*O*-*t*-butyl)hydroxymethylbenzoate (Figure 4; intermediate to 12):**

Isobutylene was liquidified in a sealed bottle at -78°C. A solution of sulfuric acid (0.5 ml) and methyl *p*-hydroxymethylbenzoate (2.00 g, 12.0 mmol) in dry ethyl ether (20 ml) was added to the isobutylene solution (8 ml) at -78°C and stirred overnight at room temperature. The resulting mixture was cooled to 4°C, then ice-cooled water was added. The ether layer was dried over magnesium sulfate and evaporated to give the desired product as a white solid (2.59 g, 96.8 %): m.p 34-36°C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 1.29 (s, 9H), 3.89 (s, 3H), 4.49 (s, 2H), 7.40 (d, *J* = 6.7 Hz, 2H), 7.98 (d, *J* = 6.7 Hz, 2H); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>) δ 27.5, 51.8, 63.4, 73.6, 126.8, 128.7, 129.5; HRMS (FAB) calcd for [C<sub>13</sub>H<sub>18</sub>O<sub>5</sub> + Cs<sup>+</sup>] 355.0310, found 355.0323.

20

25

30

35

**Synthesis of *p*-(*O*-Butyl)hydroxymethylbenzoic acid (12; Figure 4):**

Methyl *p*-(*O*-*t*-butyl)hydroxymethylbenzoate (2.02 g,

- 24 -

9.10 mmol) was dissolved in a 0.8 M LiOH solution in methanol and H<sub>2</sub>O (34 ml; methanol: H<sub>2</sub>O, 3: 1). The reaction mixture was stirred until the starting material disappeared as judged by TLC (methylene chloride: ethyl ether = 9: 1). The reaction mixture was acidified by the addition of 1N-HCl and extracted with methylene chloride. The methylene chloride layer was dried over magnesium sulfate and then evaporated to give the desired product as a white solid (1.72 g, 90.9 %): m.p 147-149°C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 1.39 (s, 9H), 4.50 (s, 2H), 7.42 (d, J = 6.8 Hz, 2H), 8.06 (d, J = 6.8 Hz, 2H); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>) δ 27.6, 63.6, 73.9, 127.0, 128.0, 130.3, 146.4, 171.8; HRMS (FAB) calcd for [C<sub>12</sub>H<sub>16</sub>O<sub>3</sub> + Na<sup>+</sup>] 231.0997, Found 231.0986.

#### Synthesis of MeO-PEG-Linker-Y<sup>\*</sup>G<sup>\*</sup>G<sup>\*</sup>F<sup>\*</sup>L<sup>\*</sup>:

**Attachment of *p*-(*O*-Butyl)hydroxymethylbenzoic acid (12) to MeO-PEG; [MeO-PEG-benzyl-OH (13); as illustrated in Figure 4]:**

Compound 12 (125 mg, 601 μmol), MeO-PEG (1.00 g, 200 μmol), and DMAP (611 μg, 50.0 μmol) were dissolved in methylene chloride (10 ml), and DCC (124 mg, 601 μmol; dicyclohexylchlorodiimide) was added. The resulting mixture was stirred for 12 h. The precipitated urea was filtered through celite. Diethyl ether was slowly added to the filtrate in order to precipitate the polymer. The polymer precipitate was washed with cold absolute ethanol and ether, and dried over P<sub>2</sub>O<sub>5</sub> under vacuum. This solid was dissolved in trifluoroacetic acid, and the resulting solution was stirred for 9 min at room temperature. The whole reaction mixture was poured onto an ice-cold diethyl ether solution with vigorous stirring. The precipitate was collected, washed with cold absolute ethanol and diethyl ether, and dried over P<sub>2</sub>O<sub>5</sub> under vacuum (935 mg,

- 25 -

91.1 %):  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  4.45 (t,  $J = 7$  Hz, 2H), 4.71 (s, 2H), 7.41 (d,  $J = 7$  Hz, 2H), 7.98 (d,  $J = 7$  Hz, 2H).

5      **Construction of (O-Benzyl)Tyr<sup>a</sup>-Gly<sup>a</sup>-Gly<sup>a</sup>-Phe<sup>a</sup>-Leu<sup>a</sup>-Boc on (13 to form 14 and 15; figure 4):**

          A mixture of 13 (195 mg, 38.0  $\mu\text{mol}$ ),  
pentafluorophenyl carbamate of Boc-[p-(O-  
benzyl)hydroxybenzyl]hydrazine (102 mg, 5 eq), and DMAP  
10      (23.2 mg, 5 eq) in methylene chloride (5 ml) was  
stirred for 24 h at room temperature. Diethyl ether  
was slowly added to this mixture to precipitate the  
polymer product 14. The polymer product was washed  
with absolute ethanol and diethyl ether, and dried over  
15       $\text{P}_2\text{O}_5$  under vacuum:  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  1.40 (s,  
9H), 4.45 (2H), 4.55 (2H), 5.00 (2H), 5.15 (2H), 6.55  
(1H), 6.88 (2H), 7.15 (2H), 7.38 (7H), 8.00 (2H). The  
polymer 14 was dissolved in TFA/methylene chloride, and  
stirred for 30 min to remove the Boc-group.  
20      Precipitation with ether, a separate wash with absolute  
ethanol and diethyl ether followed by drying over  $\text{P}_2\text{O}_5$   
under vacuum gave the trifluoro acetate salt of [p-(O-  
benzyl)hydroxybenzyl]Tyr<sup>a</sup>-O-benzyl-PEG-OMe. This salt  
was dissolved in methylene chloride and neutralized  
25      with diisopropylethylamine (DIPEA, 1 eq). To the  
resulting mixture was added the pentafluorophenyl  
carbamate of Boc-carbazate (5 eq) and DMAP (5 eq). The  
reaction mixture was stirred for 4 h. Precipitation  
with diethyl ether followed by washing with absolute  
30      ethanol and diethyl ether, then drying over  $\text{P}_2\text{O}_5$  under  
vacuum gave the product, Boc-Gly<sup>a</sup>-(O-benzyl)-Tyr<sup>a</sup>-O-  
benzyl-PEG-OMe. Repetition of this cycle of  
deprotection, neutralization, and coupling with Gly<sup>a</sup>,  
Phe<sup>a</sup>, and Leu<sup>a</sup> produced the Leu-enkephalin azatide 15  
35      (137 mg, 62.4 % from 13):  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  0.87  
(6H), 1.42 (9H), 1.91 (1H), 4.43 (2H), 4.96 (2H), 5.12

- 26 -

(2H), 6.83 (2H), 7.13 (2H), 7.37 (7H), 7.95 (2H). The multiplicity of peaks is not described due to the peak broadening.

5     **Synthesis of Tyr<sup>a</sup>-Gly<sup>a</sup>-Gly<sup>a</sup>-Phe<sup>a</sup>-Leu<sup>a</sup>-Boc (protected intermediate of 16; Figure 4):**

Compound 15 (137 mg, 23.7  $\mu$ mol) was hydrogenated with 10 % Pd/C (100 mg) in methanol (5 ml) under a balloon containing one atmosphere of hydrogen for 4 h.  
10     All volatiles were removed in vacuo and the residue was extracted with absolute ethanol. This ethanol solution was concentrated and purified by preparative thin layer chromatography. The desired material was observed as a single band  $R_f$  = 0.4 (13.25 mg, 90.7 %, TLC solvent ,  
15     methylene chloride: methanol = 9: 1): <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD)  $\delta$  0.93 (d, J = 7 Hz, 6H), 1.43 (s, 9H), 1.47 (s, 9H), 1.95 (m, 1H), 3.27 (broad s, 2H), 4.17 & 5.19 (broad s, 2H), 4.50 (broad s, 2H), 6.76 (d, J = 6.7 Hz, 2H), 7.12 (d, J = 6.7 Hz, 2H), 7.33 (m, 5H); m/z (ESI, positive) 639 (M + Na)<sup>+</sup>, 617 (M + 1)<sup>+</sup>.  
20

**Synthesis of Tyr<sup>a</sup>-Gly<sup>a</sup>-Gly<sup>a</sup>-Phe<sup>a</sup>-Leu<sup>a</sup>.2CF<sub>3</sub>COOH (16; Figure 4):**

Tyr<sup>a</sup>-Gly<sup>a</sup>-Gly<sup>a</sup>-Phe<sup>a</sup>-Leu<sup>a</sup>-Boc (13.25 mg, 21.5  $\mu$ mol)  
25     was dissolved in TFA/methylene chloride (5 ml) and stirred for 30 min. All volatiles were removed in vacuo to give the desired product as a white hygroscopic solid (16.0 mg, 100 %): <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD)  $\delta$  1.05 (d, J = 6.7 Hz, 6H), 2.09 (m, 1H), 3.07 (broad s, 2H), 4.22 & 5.26 (broad s, 2H), 4.65 (broad s, 2H), 6.77 (d, J = 6.8 Hz, 2H), 7.17 (d, J = 6.8 Hz, 2H), 7.35 (m, 5H); m/z (ESI, positive) 539 (M + Na)<sup>+</sup>, 517 (M + 1)<sup>+</sup>.  
30

35     **Azatide (16) Competition ELISA for Anti- $\beta$ -Endorphin Monoclonal Antibody.**

- 27 -

Each well of a Costar 96-well plate that was used in the competition was initially coated with 25  $\mu$ l of Tyr-Gly-Gly-Phe-Leu-CO-NH-(CH<sub>2</sub>)<sub>2</sub>-NH-CO-(CH<sub>2</sub>)<sub>2</sub>-SS-BSA (5-20 mg/ml) in 60 mM sodium bicarbonate/30 mM sodium carbonate, pH 9.3, overnight. The wells were washed ten times with deionized water and blocked with 100  $\mu$ l of 3 % BSA (all in PBS with 0.5 % Tween) to prevent nonspecific adsorption. After incubating for 30 min at 37°C in moist chamber, the 3 % BSA was then shaken out and 25  $\mu$ l of 3 % BSA and 25  $\mu$ l of 16 (competing antigen) were added to first well and serially diluted across plate; the same process was then continued in first well of second row. Well 12 was used as the positive control. The anti- $\beta$ -endorphin antibody (diluted in 1% BSA/ PBS with 0.5% Tween) was added to each well (25  $\mu$ l) and the plate was incubated at 37°C for 2 hours. The plate was washed 20 times with deionized water, and 25  $\mu$ l of a 1:1000 dilution of goat anti-mouse IgG glucose oxidase conjugate (Cappel) in 1 % BSA was added to each well and the plate was incubated at 37°C for 1 hour. The plates were washed 20 times with deionized water and bound antibody was detected by the addition of 50  $\mu$ l of developing agent [0.6 ml 20 % glucose, 40  $\mu$ l 92 mM 2,2'-azinobis(3-ethylbenzthiazolinesulfonate), and 40  $\mu$ l of 25 $\mu$ M horseradish peroxidase in 5 ml of phosphate buffer, pH 6.0] to each well. Thirty minutes later the plates were read at 405 nm.

**Synthesis of protecting group: bispentafluorophenol carbonate (1) as shown in Figure 3:**

Pentafluorophenol (0.27 mol.; commercially available from Aldrich chemical) was dissolved in 0.5 Molar KOH and cooled to 0°C. Phosgene was then passed through this solution with vigorous mixing. The pH of the reaction mixture was controlled to be no less than

- 28 -

6.0. Sometimes the carbonate crystallized from solution, but more often an oily precipitate formed. Next, the reaction mixture was kept at 0°C overnight. The solidified residue was filtered off, washed with water and dissolved in chloroform. The solution was dried over anhydrous sodium sulfate, filtered and evaporated. The crude crystalline product, with a strong, chloroformate-like odor from an impurity, was recrystallized from hexane. The yield was approximately 75%, starting with 55 grams pentafluorophenol.

**General synthesis of Compound 12 (Figures 4 and 8):**

To a solution of methyl 4-(hydroxymethyl)benzoate (2.0 g, 12mmol, 1.0 equivalent, Aldrich) in .10 Molar diethylether, was bubbled 8 mL of isobutylene (2-methylpropene, commercially available from Aldrich) at - 78°C. Next, 10 drops of sulfuric acid were added and the mixture was allowed to stir overnight. The reaction mixture was diluted with ether (25 mL), quenched with sodium bicarbonate (10 mL), washed with water (10 mL), condensed and dried over magnesium sulfate. The product can be purified by flash chromatography or distillation. The product is next exposed to 5 equivalents of LiOH·H<sub>2</sub>O in a 3:1 mixture of methanol water (3 Molar). The mixture is allowed to stir for 2 hours at 25°C and then is extracted with ether and acidified with 1 mL of HCl. The precipitate is collected on a glass filter and can be further purified by flash chromatography or crystallization.

**Synthesis of compound 13 (Figure 8):**

To a solution of (MeO-PEG-OH, n = 5000 MW, commercially available from Sigma Company) in 17 mM of methylene chloride at 25°C, is added 3.0 equivalents of compound 12, 3.0 equivalents of 1,3 dicyclohexyl

- 29 -

carbodiimide (DCC) and .75 equivalents of 4-DMAP (4-dimethylaminopyridine). The reaction mixture is allowed to stir overnight. Next, the mixture is exposed to 3.0 equivalents of trifluoroacetic acid (TFA) and allowed to stir an additional 11 minutes at 25°C. The mixture is then poured into ice-cold ether (approximately 17 mM) to precipitate the PEG and then washed with cold ether and ethanol fractions. The final product can be further purified by crystallization from hot ethanol.

**First coupling of the activated azacarbamate addition molecule to the PEG support (Figure 8): synthesis of Compound 17 (library of compounds with chain length 1):**

To 1.0 equivalent of the PEG support (compound 13) in 17 mM methylene chloride at 25°C (n-reaction vessels; wherein  $1 \leq n \leq 100$ ), is added 5.0 equivalents of the activated azacarbamate (addition molecule-activated azacarbamate residues synthesized *infra* wherein  $R_x$  is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl, benzyl, and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val. - combinatorial strategy in figure 8; the "n"th-addition molecule is added separately to the "n"th reaction vessel which contains the PEG support) and 5.1 equivalents of 4-dimethylaminopyridine (4-DMAP; added to n reaction vessels). In each vessel, the reaction mixture is then allowed to stir for 24 hours and is next precipitated with the addition of ether (17 mM diethyl ether). The product is then further purified by washing with ether (1X) and crystallizing in cold ethanol (1X). 1/3 of the products of each "n" reaction vessels are saved and the remainder 2/3 of products are recombined and mixed into a single reaction vessel to form a library of

- 30 -

nascent protected compounds with chain length 1  
(compound 17).

**Synthesis of Compound 18: Removal of Boc group (Figure 8):**

1.0 gram of compound 18 is exposed to a 10% trifluoroacetic acid/ methylene chloride solution (10 mL, 1:1 TFA/ methylene chloride) and allowed to stir at 25°C for 1 hour. The reaction mixture is next precipitated with the addition of ether (17 mM diethyl ether). The product is then further purified by washing with ether (1X) and can be crystallized from ethanol (1X).

**Second coupling of the activated azacarbamate addition molecule to the PEG support (Figure 9): synthesis of Compound 19 (library of compounds with chain length 2):**

To 1.0 equivalent of the compound 18 in 17 mM methylene chloride at 25°C (n-reaction vessels; wherein  $1 \leq n \leq 100$ ), is added 5.0 equivalents of the activated azacarbamate (addition molecule-activated azacarbamate residues *synthesized infra* wherein  $R_x$  is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl, benzyl, and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val combinatorial strategy in figure 9; the "n"th-addition molecule is added separately to the "n"th reaction vessel which contains compound 18) and 5.1 equivalents of 4-dimethylamino pyridine (4-DMAP) is added to each of the n reaction vessels. In each vessel, the reaction mixture is then allowed to stir for 24 hours and is next precipitated with the addition of ether (17 mM diethyl ether). The product is then further purified by washing with ether (1X) and crystallizing in cold ethanol (1X). 1/3 of

- 31 -

the products of each "n" reaction vessels are saved and the remainder 2/3 of products are recombined and mixed into a single reaction vessel to form a library of nascent protected compounds with chain length 2 (compound 19).

**Synthesis of Compound 7: Removal of Boc group (Figure 9):**

1.0 gram of compound 19 is exposed to a 10% trifluoroacetic acid/ methylene chloride solution (10 mL, 1:1 TFA/ methylene chloride) and allowed to stir at 25°C for 1 hour. The reaction mixture is next precipitated with the addition of ether (17 mM diethyl ether). The product is then further purified by washing with ether (1X) and can be crystallized from ethanol (1X).

Compound 22 is formed from an iterative cycle of steps 1-3 as outlined below and illustrated in Figure 10:

**Step 1: 'nth' coupling of the activated azacarbamate to the PEG support (as illustrated in Figure 10):**

To 1.0 equivalent of the nascent deprotected chain of length 2 (compound 20) in 17 mM methylene chloride at 25°C (n-reaction vessels; wherein  $1 \leq n \leq 100$ ), is added 5.0 equivalents of the activated azacarbamate (see boxed addition molecule-activated azacarbamate residues synthesized *infra* wherein  $R_x$  is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl, benzyl, and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val- combinatorial strategy in figure 10; the "n"th-addition molecule is added separately to the "n"th reaction vessel which contains the PEG support) and 5.1 equivalents of 4-

- 32 -

dimethylaminopyridine (4-DMAP; added to n reaction vessels). In each vessel, the reaction mixture is then allowed to stir for 24 hours and is next precipitated with the addition of ether (17 mM diethyl ether). The product is then further purified by washing with ether (1X) and crystallizing in cold ethanol (1X). 1/3 of the products of each "n" reaction vessels are saved and the remainder 2/3 of products are recombined and mixed into a single reaction vessel to form a library of nascent protected compounds with chain length n.

Step 2: 'nth' removal of Boc group (Figure 10)  
1.0 gram of the azatide polymer formed in step 1 is exposed to a 10% trifluoroacetic acid/ methylene chloride solution (10 mL, 1:1 TFA/ methylene chloride) and allowed to stir at 25°C for 1 hour. The reaction mixture is next precipitated with the addition of ether (17 mM diethyl ether). The product is then further purified by washing with ether (1X) and can be crystallized from ethanol (1X).

Step 3: Repeat steps i-ii as desired:  
Steps i-ii (supra) may be reiterated as many times as desired to create azatide polymers wherein  $1 \leq n \leq 100$ .

Synthesis of Compound 22: final deprotection step (figure 10):

This step removes the aza-peptide from the PEG support and additionally removes the benzyl, benzyl ester, 9-fluorenylmethyl and S-sulphenylthiocarbonate protecting groups: To 1.0 gram of azatide polymer in 10 mL methanol at 25°C is added 200 mg of 10% Pd/C. The reaction mixture is capped with a hydrogen balloon and allowed to stir overnight. The product is washed with ether, filtered and condensed. Further purification can be achieved by standard chromatographic

- 33 -

methodologies for small azatides or crystallization for large polymers.

Removal of the remaining t-But protecting group:  
5 1.0 gram of azatide polymer is exposed to a 10% trifluoroacetic acid/ methylene chloride solution (10 mL, 1:1 TFA/ methylene chloride) and allowed to stir at 25°C for 1 hour. The product is washed with ether, washed with sodium bicarbonate, dried over sodium  
10 sulfate and condensed. Further purification can be achieved by standard chromatographic methodologies for small peptides.

**Synthesis of azatide residue with Ala-R-group (figure 11; equation 1):**  
15

A solution of 0.098 mole of methyl-iodide in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55  
20 Molar of ethanol. After a reflux period of 6 hours, the ethanol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalent, was next exposed to 1.1 equivalents of  
25 di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried  
30 over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0  
35 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40

- 34 -

minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Val-R-group (figure 11; equation 2):**

A solution of 0.098 mole of 2-chloropropane in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the alcohol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalents, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate

- 35 -

is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

5       **Synthesis of azatide residue with Leu-R-group (figure 11; equation 3):**

          A solution of 0.098 mole of 1-chloro-2-methylpropane (from Aldrich company) in 0.98 Molar of ethanol was added over a period of 1 hour to a  
10       refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the alcohol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried  
15       with potassium carbonate and filtered. The crude base, 1.0 equivalent, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next  
20       removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

25         
          Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1  
30       equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard  
35       purification methodologies.

- 36 -

**Synthesis of azatide residue with Ile-R-group (figure 11; equation 4):**

A solution of 0.098 mole of 2-chlorobutane (from Aldrich company) in 0.98 Molar of ethanol was added  
5 over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the alcohol was removed by distillation. The residue was extracted with ether,  
10 the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalents, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then  
15 stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a  
20 methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40  
25 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography,  
30 distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Pro-R-group (figure 11; equation 5):**

35 A solution of 0.098 mole of pyrazolidine (from Aldrich company) in 0.98 Molar of ethanol was added

- 37 -

over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the alcohol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalent, was next exposed to 1.1 equivalents of di-*tert*-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Phe-R-group (figure 12; equation 6):**

A solution of 0.098 mole of benzyl chloride in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the alcohol was removed by distillation. The

- 38 -

residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalent, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Trp-R-group (figure 12; equation 7):**

A solution of 0.098 mole of chloro-3-methyl-indole (Aldrich company) in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the alcohol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalent, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate

- 39 -

(commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Met-R-group (figure 12; equation 8):**

A solution of 0.098 mole of 1-chloro-2-thiomethyl-ethane (Aldrich company) in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the alcohol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalent, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was

- 40 -

extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

5

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

15

**Synthesis of azatide residue with Gly-R-group (figure 12; equation 9):**

A solution of 85% hydrazine hydrate (10 equivalents; commercially available from Aldrich company) in 2.55 Molar of ethanol was exposed to 1.0 equivalent of di-tert-butyl dicarbonate (commercially available from Aldrich company) and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

25

30

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar

35

- 41 -

methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

5

**Synthesis of azatide residue with Ser-R-group (figure 12; equation 10):**

Formation of  $\text{BnO-CH}_2\text{Cl}$ , used infra, is accomplished by the dropwise addition of 1.1 equivalents of preformed  $\text{NaOCH}_2\text{Ph}$  (formed by the addition of 1.1 equivalents  $\text{NaH}$  to 1.0 equivalent benzylalcohol in methylene chloride (1.0 M) at 0°C; 1 hour) to bromochloromethane (Aldrich) in methylene chloride (1.0 M) at 0°C for 1 hour. The reaction mixture is then quenched with ammonium chloride, washed with water and further purified by flash chromatography to afford  $\text{BnO-CH}_2\text{Cl}$ .

A solution of 0.098 mole of  $\text{BnO-CH}_2\text{Cl}$  (synthesized supra) in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the alcohol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalent, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

- 42 -

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Thr-R-group (figure 13; equation 11):**

Formation of  $\text{BnO-CHCl-CH}_3$ , used infra, is accomplished by the dropwise addition of 1.1 equivalents of preformed  $\text{NaOCH}_2\text{Ph}$  (formed by the addition of 1.1 equivalents  $\text{NaH}$  to 1.0 equivalent benzylalcohol in methylene chloride (1.0 M) at 0°C; 1 hour) to 1,1-dichloroethane (Aldrich) in methylene chloride (1.0 M) at 0°C for 1 hour. The reaction mixture is then quenched with ammonium chloride, washed with water and further purified by flash chromatography to afford  $\text{BnO-CHCl-CH}_3$ .

A solution of 0.098 mole of  $\text{BnO-CHCl-CH}_3$  (synthesized supra) in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the alcohol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalents, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then

- 43 -

stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Cys-R-group (figure 13; equation 12):**

Formation of  $\text{HSCH}_2\text{Cl}$  is accomplished by the dropwise addition of 1.1 equivalents of preformed NaSH (Aldrich) to bromochloromethane (Aldrich) in methylene chloride (1.0 M) at 0°C for 1 hour. Next, a solution of 0.098 mole of  $\text{HSCH}_2\text{Cl}$  (in situ, synthesized supra) in 0.98 Molar of ethanol is added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the alcohol is removed by distillation. The residue is extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalents, is next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The

- 44 -

solvent is next removed by distillation and the remaining residue is extracted with ether, ished in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 0°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

To protect the sulfydryl group, an S-sulfenylthiocarbonate derivative is made according to the procedure of Nokihara et al. *J Org. Chem.*, 43, 4893 (1978) which forms the protected azatide residue after standard purification methodologies.

**Synthesis of azatide residue with Tyr-R-group (figure 13; equation 13):**

To a solution of 1.0 equivalent of p-hydroxybenzylbromide in methylene chloride is added 1.1 equivalents of 60% sodium hydride at 0°C and allowed to stir for 1 hour. Next, 1.1 equivalents of benzyl bromide is added and the mixture is allowed to stir overnight. The mixture is then quenched with water, diluted with ether and purified by distillation. 1.0 equivalents of the compound is next added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich

- 45 -

company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the alcohol is removed by distillation. The residue is extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalents, is next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent is next removed by distillation and the remaining residue is extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Asn-R-group (figure 13; equation 14):**

Formation of 2-chloroacetyl-N-9-fluorenylmethylcarbamate, used infra, is formed from protection of 1.0 equivalent 2-chloro-acetamide (Aldrich) with 1.1 equivalents 9-fluorenyl-CH<sub>2</sub>OCOC1 (Aldrich) in 0.10 M methylene chloride at 0°C for 1 hour. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

- 46 -

Next, a solution of 0.098 mole of 2-chloroacetyl-N-9-fluorenylmethylcarbamate (vida supra) in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the ethanol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalent, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Gln-R-group (figure 13; equation 15):**

Formation of 3-chloroethyl-N-9-fluorenylmethylcarbamate, used infra, is formed from protection of 1.0 equivalent 3-chloro-ethylamide (Aldrich) with 1.1 equivalents 9-fluorenyl-CH<sub>2</sub>OCOC1

- 47 -

(Aldrich) in 0.10 M methylene chloride at 0°C for 1 hour. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

5

Next, a solution of 0.098 mole of 3-chloroethyl-N-9-fluorenylmethylcarbamate (vida supra) in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the ethanol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalent, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

25

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

35

Synthesis of azatide residue with Asp-R-group (figure

- 48 -

**14; equation 16):**

Formation of Bn-O<sub>2</sub>CCH<sub>2</sub>-OTf, used infra, is formed from protection of 1.0 equivalent benzylglycolate (Aldrich) with 1.1 equivalents trifluoroacetic anhydride (Aldrich) and 1.1 equivalents triethylamine in 0.10 M methylene chloride at 0°C for 1 hour. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Next, a solution of 0.098 mole of Bn-O<sub>2</sub>CCH<sub>2</sub>-OTf (vida supra) in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the ethanol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalents, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography,

- 49 -

distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Glu-R-group (figure 14; equation 17):**

5                    Formation of  $\text{Bn-O}_2\text{CCH}_2\text{CH}_2\text{-OTf}$ , used infra, is formed from protection of 1.0 equivalent benzyl-3-hydroxy-propanoate (Aldrich) with 1.1 equivalents trifluoroacetic anhydride (Aldrich) and 1.1 equivalents  
10                   triethylamine in 0.10 M methylene chloride at 0°C for 1 hour. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

15                   Next, a solution of 0.098 mole of  $\text{Bn-O}_2\text{CCH}_2\text{CH}_2\text{-OTf}$  (vida supra) in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a  
20                   reflux period of 6 hours, the ethanol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalents, was next exposed to 1.1 equivalents of di-tert-butyl  
25                   dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried  
30                   over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

35                   Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40

- 50 -

minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Lys-R-group (figure 14; equation 18):**

Formation of (9-Fluor)-NH-(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>OTf, used infra, is formed from protection of 1.0 equivalent NH<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>OH (4-hydroxybutylamine; Aldrich) with 1.1 equivalents 9-fluorenyl-CH<sub>2</sub>OCOC1 (Aldrich) in 0.10 M methylene chloride at 0°C for 1 hour. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient. Next, 1.0 equivalent of the product is reacted with 1.1 equivalents trifluoroacetic anhydride (Aldrich) and 1.1 equivalents triethylamine in 0.10 M methylene chloride at 0°C for 1 hour. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Next, a solution of 0.098 mole of (9-Fluor)-NH-(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>OTf (vida supra) in 0.98 Molar of ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the ethanol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalents, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and

- 51 -

then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with Arg-R-group (figure 14; equation 19):**

Formation of (9-Fluor)-NH-CN<sub>2</sub>H-NH-(CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>OTf, used infra, is formed from protection of 1.0 equivalent NH<sub>2</sub>-CNH-NH-(CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>OH (Aldrich) with 1.1 equivalents 9-fluorenyl-CH<sub>2</sub>OCOC<sub>2</sub>H<sub>5</sub> (Aldrich) in 0.10 M methylene chloride at 0°C for 1 hour. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient. Next, 1.0 equivalent of the product is reacted with 1.1 equivalents trifluoroacetic anhydride (Aldrich) and 1.1 equivalents triethylamine in 0.10 M methylene chloride at 0°C for 1 hour. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Next, a solution of 0.098 mole of (9-Fluor)-NH-CN<sub>2</sub>H-NH-(CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>OTf (vida supra) in 0.98 Molar of

- 52 -

ethanol was added over a period of 1 hour to a refluxing solution of 0.51 mol of 85% hydrazine hydrate (commercially available from Aldrich company) in 2.55 Molar of ethanol. After a reflux period of 6 hours, the ethanol was removed by distillation. The residue was extracted with ether, the ethereal extracts dried with potassium carbonate and filtered. The crude base, 1.0 equivalents, was next exposed to 1.1 equivalents of di-tert-butyl dicarbonate (commercially available from Aldrich company) in 0.1 Molar methylene chloride and then stirred overnight at 25°C. The solvent was next removed by distillation and the remaining residue was extracted with ether, washed in water (1X) and dried over potassium carbonate and filtered. Product can then be purified by flash chromatography using a methylene chloride: ether/petroleum ether gradient.

Formation of the activated azacarbamate: 1.0 equivalent of the above synthesized azatide residue is added dropwise via syringe pump over a period of 30-40 minutes to bispentafluorophenol carbonate (1.1 equivalent; synthesized supra) and 1.1 equivalent dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar methylene chloride at 25°C. The activated azacarbamate is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

**Synthesis of azatide residue with His-R-group (figure 14; equation 20)**

Formation of 4-trifluoroacetoxyethylimidazole-3-N-(9-fluorenylcarbamate), used infra, is formed from protection of 1.0 equivalent 4-hydroxymethylimidazole (Aldrich) with 1.1 equivalents 9-fluorenyl-CH<sub>2</sub>OCOC1 (Aldrich) in 0.10 M methylene chloride at 0°C for 1 hour. Product can then be purified by flash

- 53 -

chromatography using a methylene chloride:  
ether/petroleum ether gradient. Next, 1.0 equivalent  
of the product is reacted with 1.1 equivalents  
trifluoroacetic anhydride (Aldrich) and 1.1 equivalents  
5 triethylamine in 0.10 M methylene chloride at 0°C for 1  
hour. Product can then be purified by flash  
chromatography using a methylene chloride:  
ether/petroleum ether gradient.

10 Next, a solution of 0.098 mole of 4-  
trifluoroacetoxymethylimidazole-3-N-(9-  
fluorenylcarbamate) (vida supra) in 0.98 Molar of  
ethanol was added over a period of 1 hour to a  
refluxing solution of 0.51 mol of 85% hydrazine hydrate  
15 (commercially available from Aldrich company) in 2.55  
Molar of ethanol. After a reflux period of 6 hours,  
the ethanol was removed by distillation. The residue  
was extracted with ether, the ethereal extracts dried  
with potassium carbonate and filtered. The crude base,  
20 1.0 equivalents, was next exposed to 1.1 equivalents of  
di-tert-butyl dicarbonate (commercially available from  
Aldrich company) in 0.1 Molar methylene chloride and  
then stirred overnight at 25°C. The solvent was next  
removed by distillation and the remaining residue was  
25 extracted with ether, washed in water (1X) and dried  
over potassium carbonate and filtered. Product can  
then be purified by flash chromatography using a  
methylene chloride: ether/petroleum ether gradient.

30 Formation of the activated azacarbamate: 1.0  
equivalent of the above synthesized azatide residue is  
added dropwise via syringe pump over a period of 30-40  
minutes to bispentafluorophenol carbonate (1.1  
equivalent; synthesized supra) and 1.1 equivalent  
35 dimethylaminopyridine (DMAP; Aldrich) in 0.10 Molar  
methylene chloride at 25°C. The activated azacarbamate

- 54 -

is then further purified by flash chromatography, distillation or crystallization by standard purification methodologies.

- 55 -

What is claimed is

1. A process for synthesizing an oligoazatide comprising the following steps:

5 Step A: providing a soluble homopolymer support with a linker unit attached to said soluble homopolymer support; then

Step B: providing a Boc-protected aza-amino acid; then

10 Step C: reacting the Boc-protected aza-amino acid of said Step B with a carbonyl activation element for producing an activated carbamate of the Boc-protected aza-amino acid; then

15 Step D: coupling the soluble homopolymer support of said Step A with the activated carbamate of said Step C for producing a nascent protected chain; then

20 Step E: deprotecting the nascent protected chain of said Step D using a mild acid for producing a nascent deprotected chain; then

Step F: washing the nascent deprotected chain by precipitation of the soluble homopolymer.

25 Step G: extending the nascent deprotected chain of said Step E by repeating steps A through F "n" times wherein  $1 \leq n \leq 100$  and wherein the soluble homopolymer support of said Step A is replaced with the nascent deprotected chain of said Step E for producing an extended deprotected chain; and then

30 Step H: decoupling and separating the extended deprotected chain of said Step F from the soluble support by hydrogenation and precipitation for producing the oligoazatide.

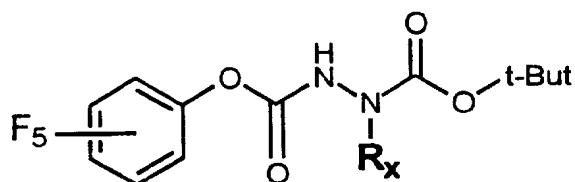
- 56 -

2. A process for synthesizing an oligoazatide as described in claim 1 wherein after said Step E:

in said Step A, the soluble homopolymer support is polyethylene glycol monomethyl ether (MeO-PEG).

3. A process for synthesizing an oligoazatide as described in claim 1 wherein:

in said Step B, said Boc-protected aza-amino acid is represented by the following structure:



wherein R<sub>x</sub> is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl, C<sub>1</sub>-C<sub>6</sub> alkyl, benzyl, substituted benzyl and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val; and

in said Step C, the carbonyl activation element is bis-pentafluorophenyl carbonate.

4. A process for synthesizing an oligoazatide as described in claim 1 wherein:

in said Step A, said linker unit is *p*-hydroxymethylbenzoate.

5. A process for synthesizing an oligoazatide as described in claim 1 wherein:

in said Step E, said mild acid is trifluoroacetic acid.

- 57 -

6. A process for producing a combinatorial oligoazatide library comprising the following steps:

Step A: providing a soluble homopolymer support with a linker unit attached to said soluble homopolymer support in each of "n" reaction vessels; then

Step B: providing "n" Boc-protected aza-amino acids; then

Step C: reacting each of the "n" Boc-protected aza-amino acid of said Step B with a carbonyl activation element for producing an activated carbamate of the Boc-protected aza-amino acids; then

Step D: adding one of the "n" activated carbamates of said Step C to each of the "n" reaction vessels and coupling the soluble homopolymer support of said Step A with the activated carbamate of said Step C for producing a nascent protected chain in each of the "n" reaction vessels; then

Step F: washing each of the nascent protected chain by precipitation of the soluble homopolymer;

Step G: saving and cataloging an aliquot from each of the "n" reaction vessels; then

Step H: pooling all "n" of the reaction vessels into a common pot for forming a mixture of nascent protected chains; then

Step I: deprotecting the nascent protected chain in each of the "n" reaction vessels using a mild acid for producing nascent deprotected chains; then

Step J: washing each of the nascent deprotected chains by precipitation of the soluble homopolymer;

Step K: equally aliquoting the mixture of nascent deprotected chains from the common pot into the

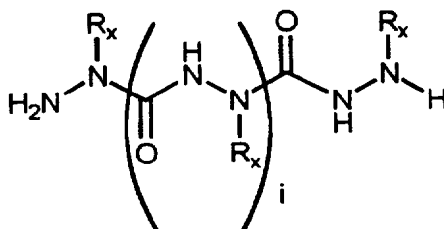
- 58 -

"n" reaction vessels; then

Step L: extending the nascent deprotected chains of said Step K by repeating steps A through J "m" times wherein  $1 \leq m \leq 100$  and wherein the soluble homopolymer support of said Step A is replaced with the nascent deprotected chain of said Step J for producing extended deprotected chains; and then

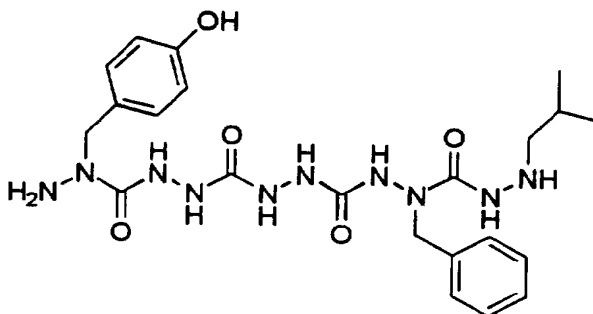
Step M: decoupling and separating the extended deprotected chains in each of the "n" reaction vessels of said Step K from the soluble support by hydrogenation and precipitation for producing the combinatorial oligoazatide library.

7. A combinatorial oligoazatide library comprising a plurality of compounds represented by the following formula:

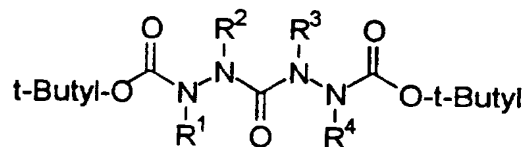


wherein  $0 \leq i \leq 99$  and  $\text{R}_x$  is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl,  $\text{C}_1$ - $\text{C}_6$  alkyl, benzyl, substituted benzyl and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

8. An azatide compound represented by the following formula:



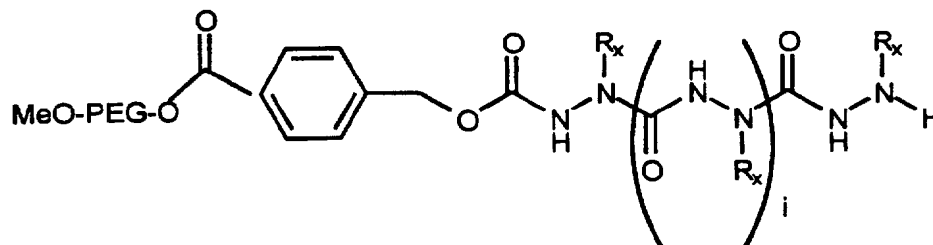
5        9.    An azatide compound represented by the following  
         formula:



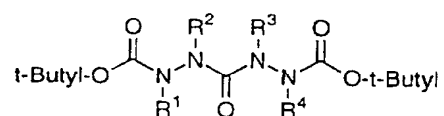
10 wherein R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> and R<sub>4</sub> is selected from the group consisting of hydrogen, methyl, isopropyl, isobutyl and benzyl.

- 60 -

10. An intermediate oligoazatide compound represented by the following formula:



5 wherein  $0 \leq i \leq 99$  and  $R_x$  is selected from the group consisting of hydrogen, methyl, isobutyl, isopropyl,  $C_1$ - $C_6$  alkyl, benzyl, substituted benzyl and the side chain radical of the following amino acids: Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, 10 Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.



Compound	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>	Yield (%)
5	H	H	H	H	92
6	Methyl	H	H	Methyl	91
7	H	Methyl	H	Methyl	90
8	H	Methyl	H	Benzyl	85
9	H	Methyl	H	Isobutyl	84
10	H	Isobutyl	H	Isobutyl	82
11	H	Isopropyl	H	Isopropyl	84

FIGURE 1

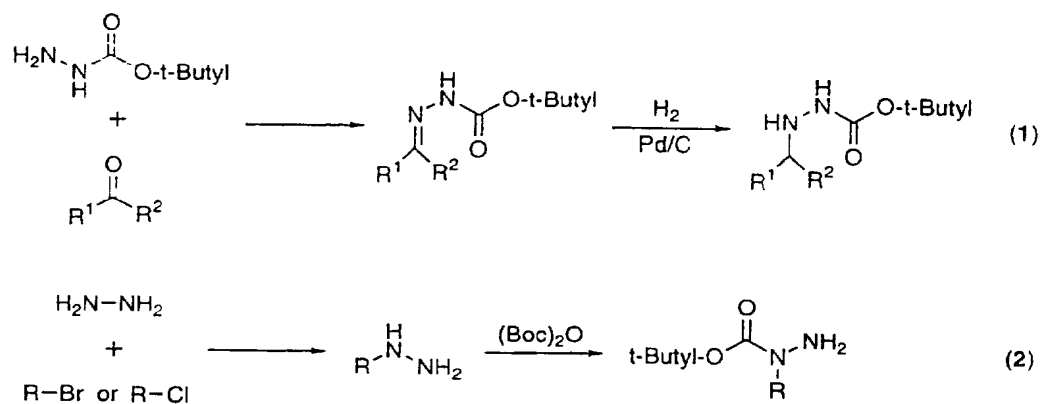
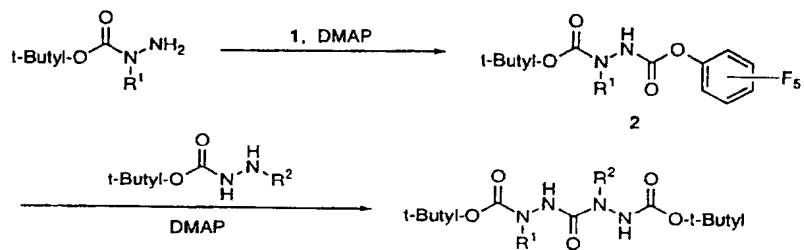


FIGURE 2

1. Starting from 1-R<sup>1</sup>-Hydrazine Carboxylic Acid, 1,1-Dimethylethyl Ester:



2. Starting from 2-R<sup>1</sup>-Hydrazine Carboxylic Acid, 1,1-Dimethylethyl Ester:

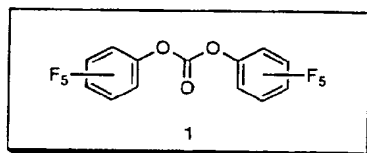
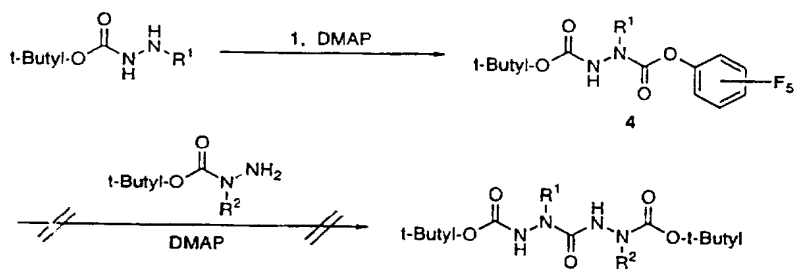


FIGURE 3

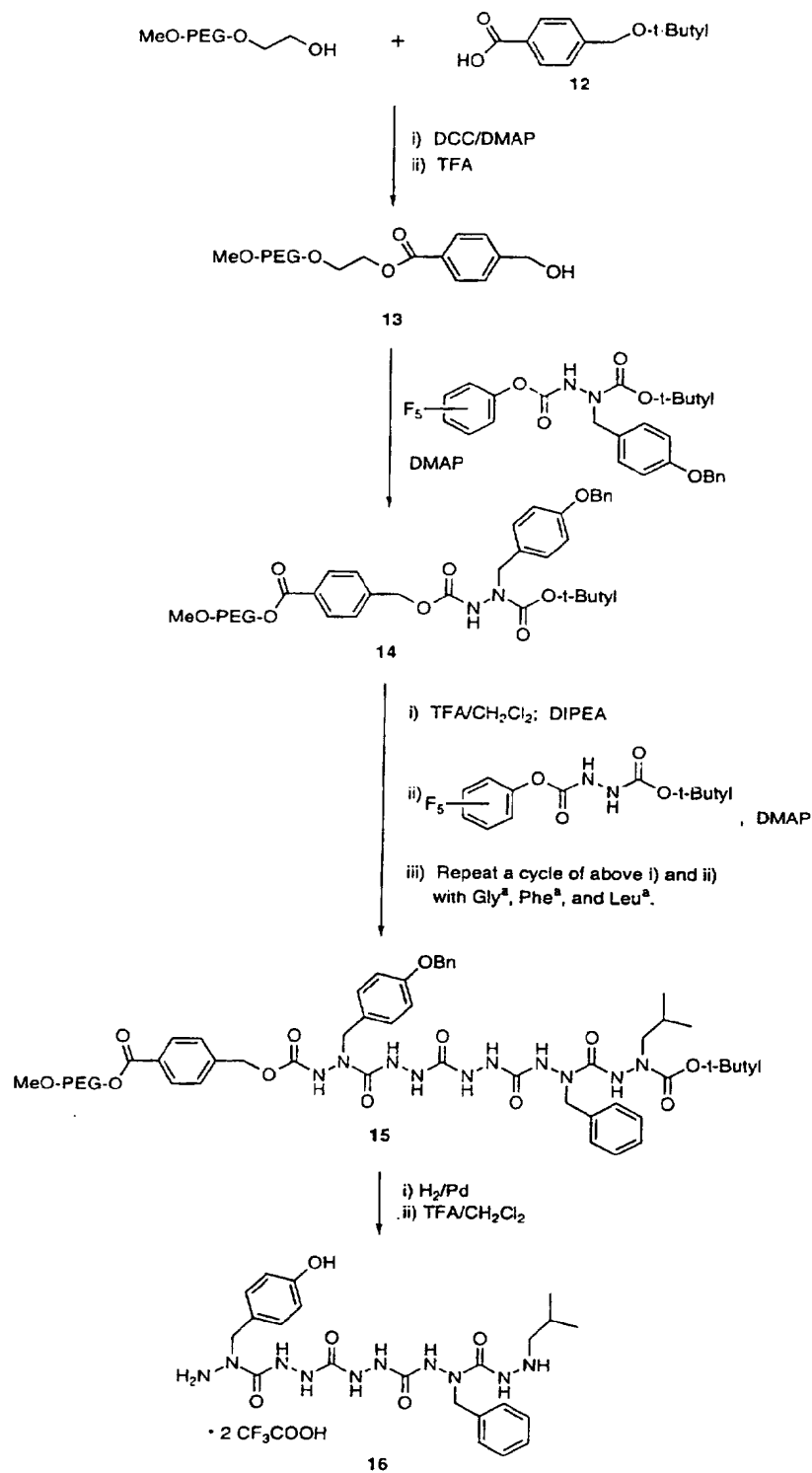
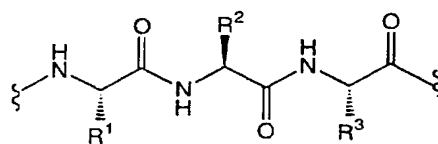
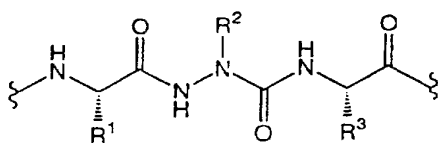


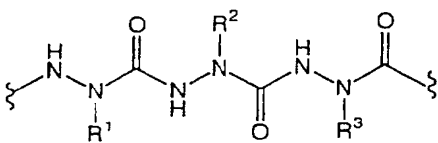
FIGURE 4



Peptide



Azapeptide



Azatide

FIGURE 5

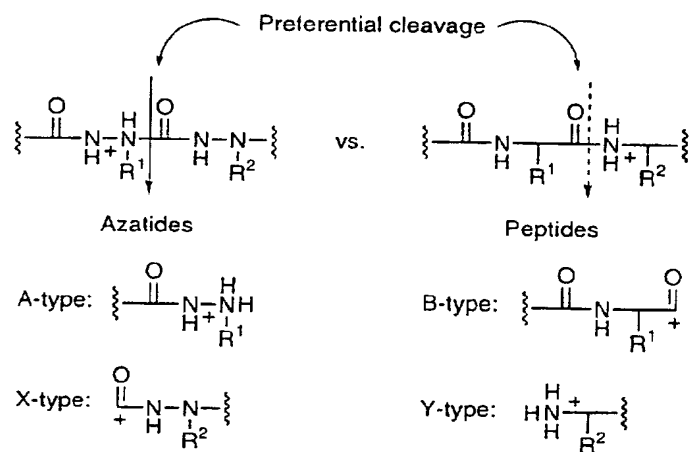


FIGURE 6

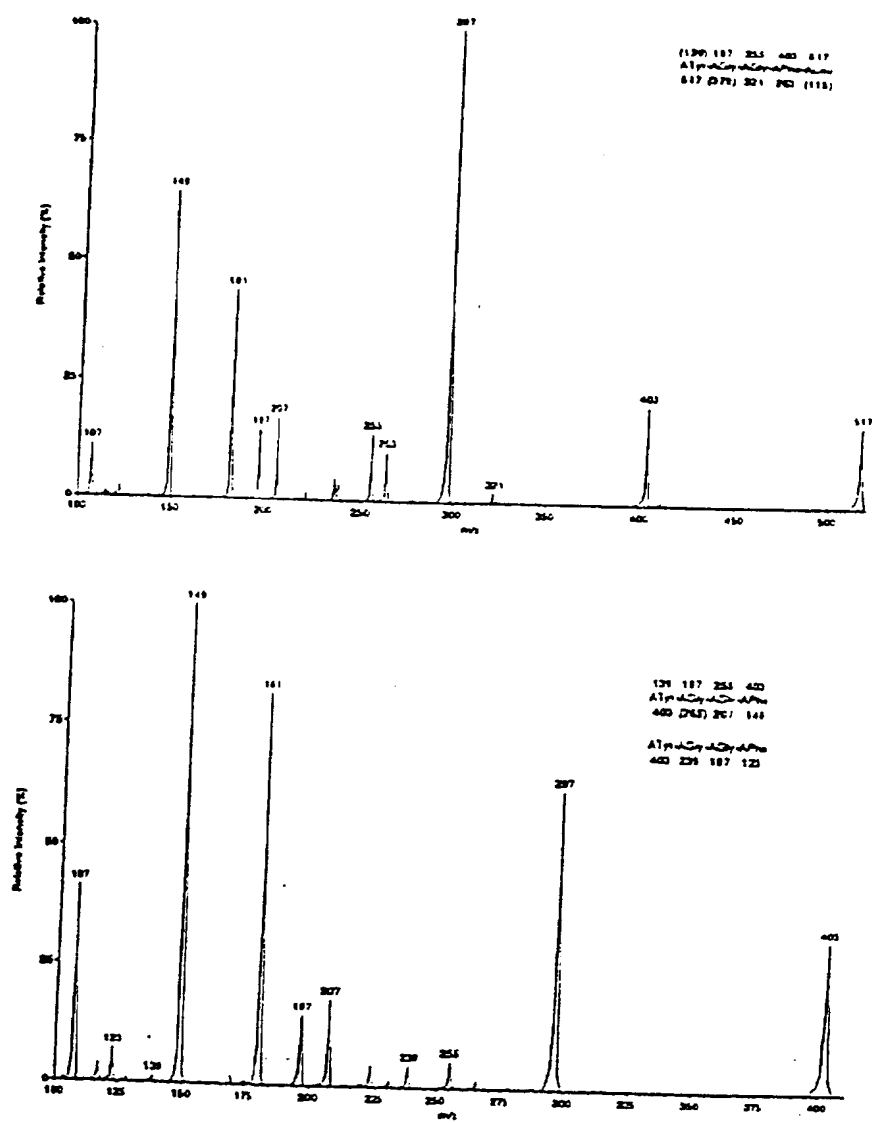


FIGURE 7

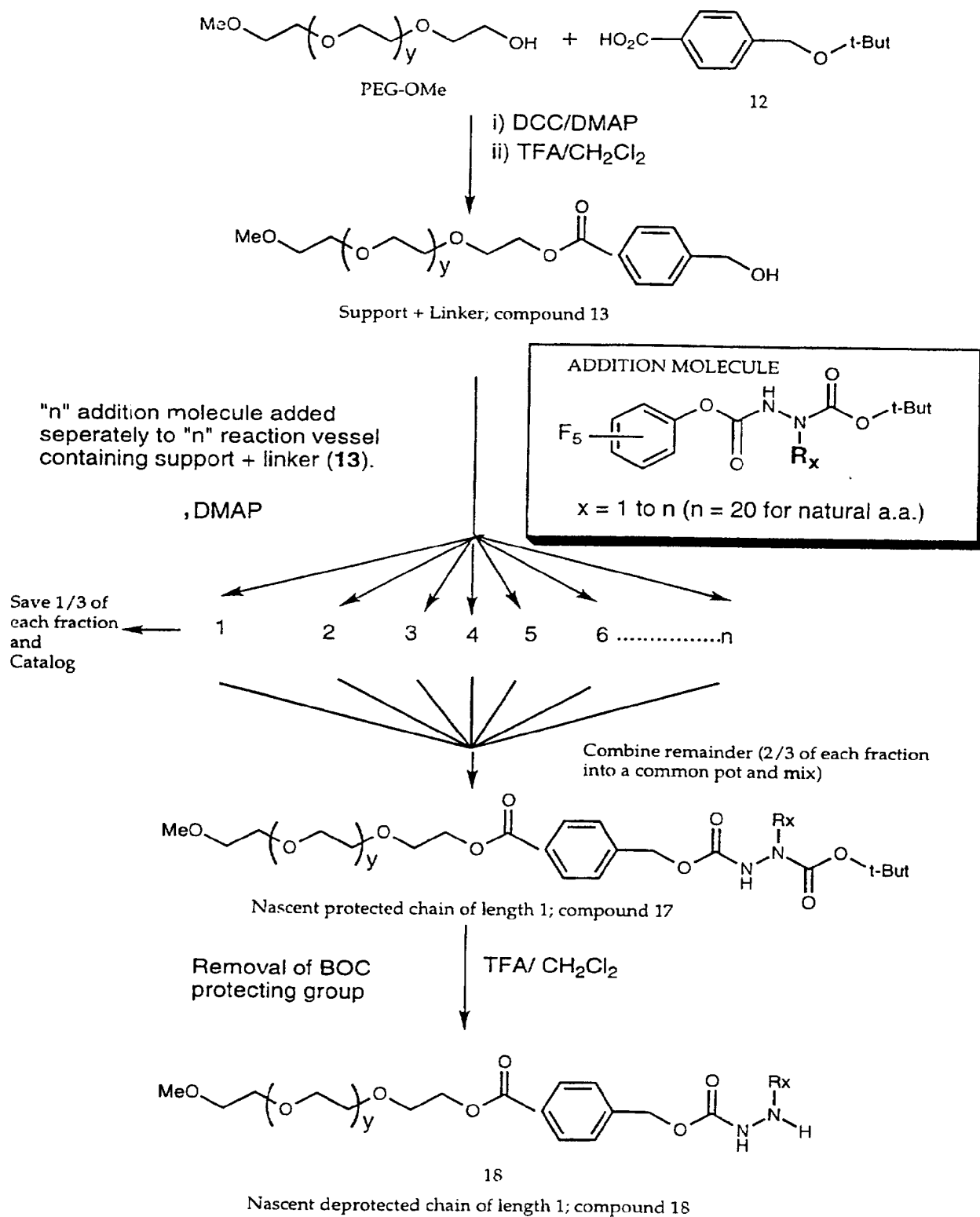


FIGURE 8

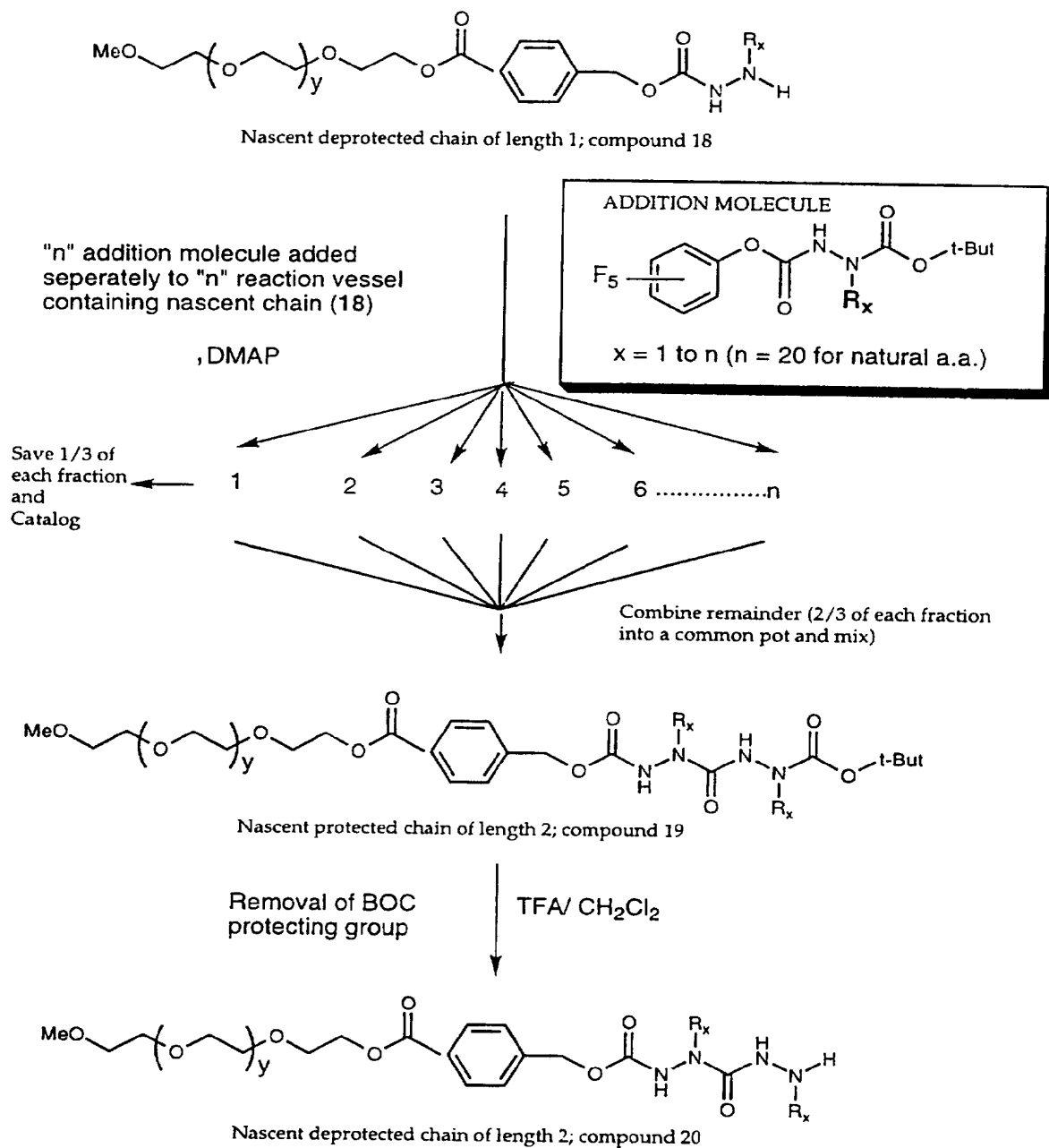
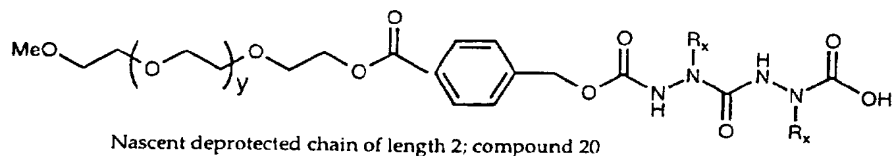


FIGURE 9



Nascent deprotected chain of length 2; compound 20

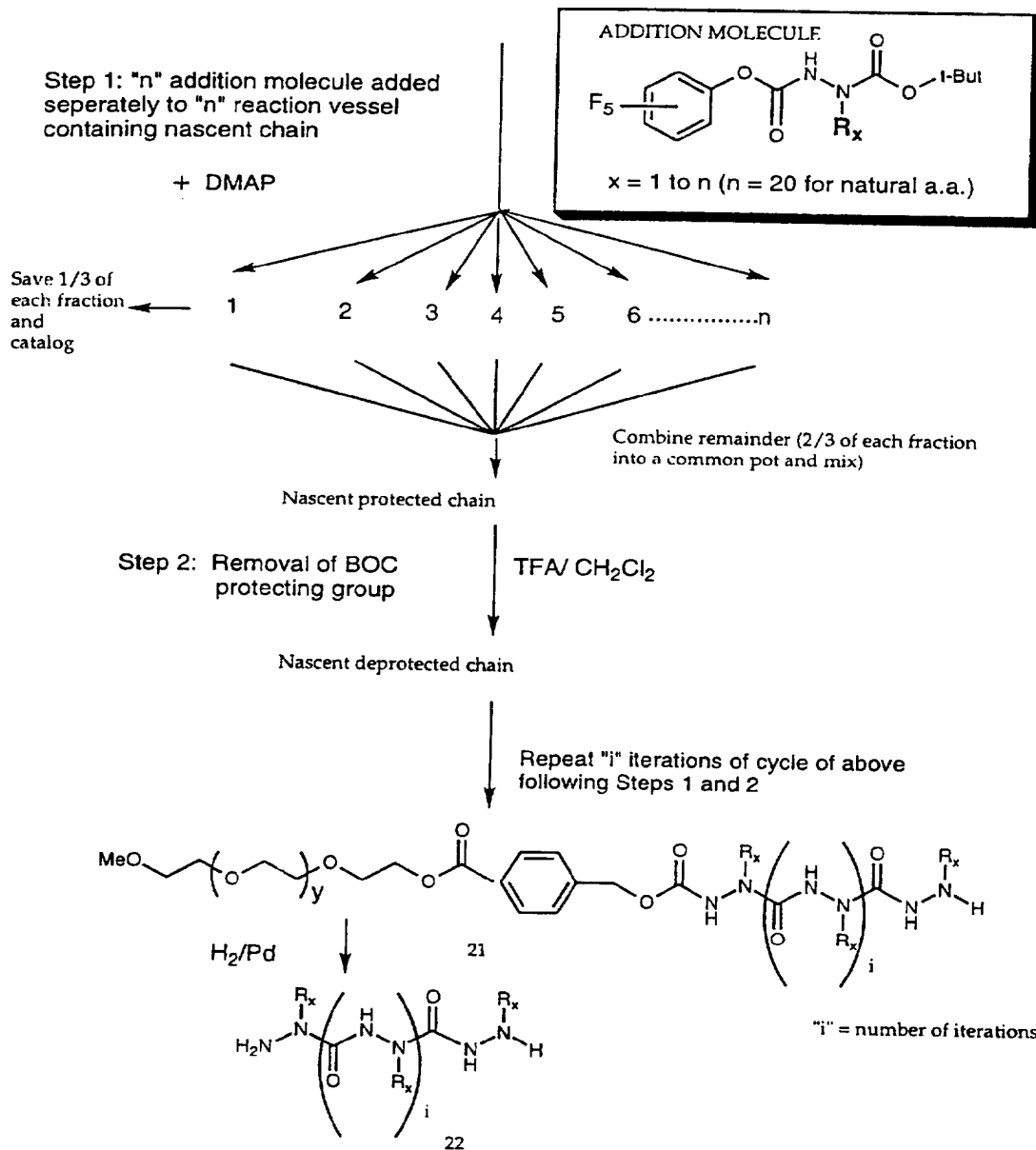
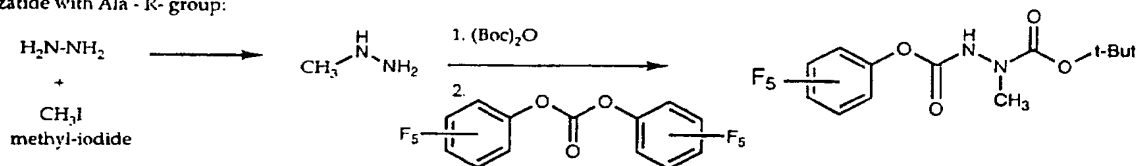
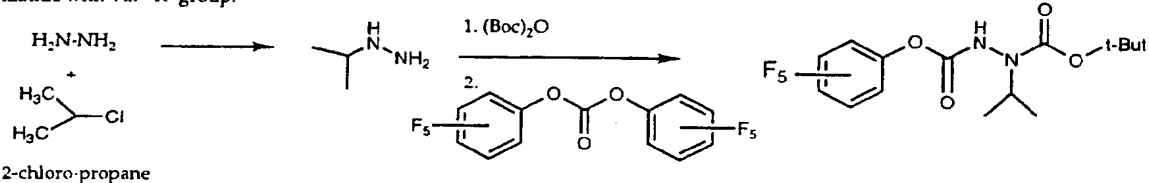


FIGURE 10

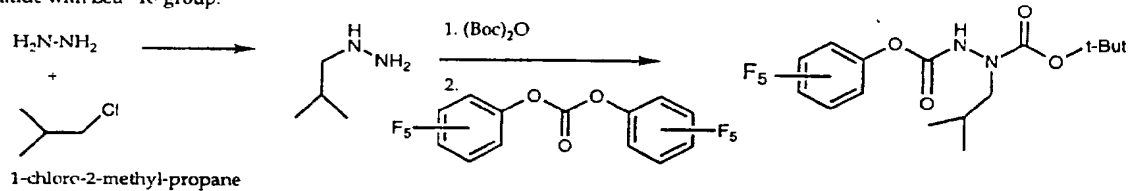
## 1. Azatide with Ala - R- group:



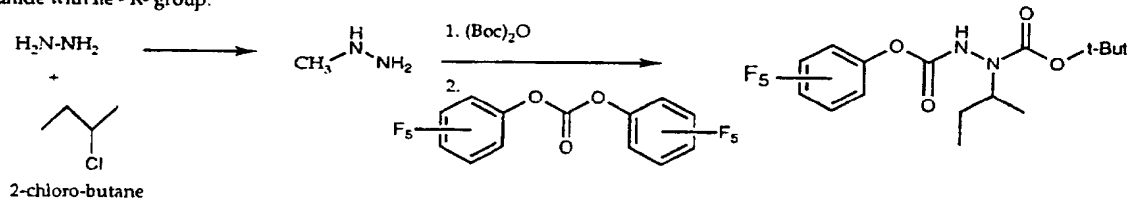
## 2. Azatide with Val - R- group:



## 3. Azatide with Leu - R- group:



## 4. Azatide with Ile - R- group:



## 5. Azatide with Pro - R- group:

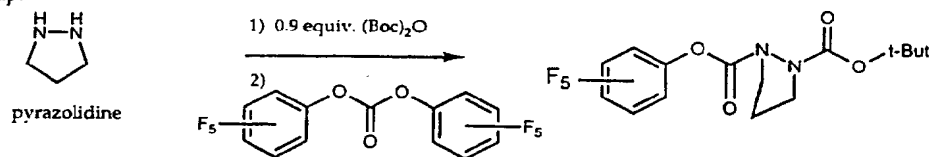
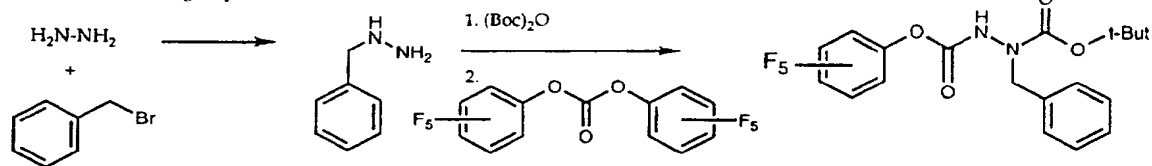
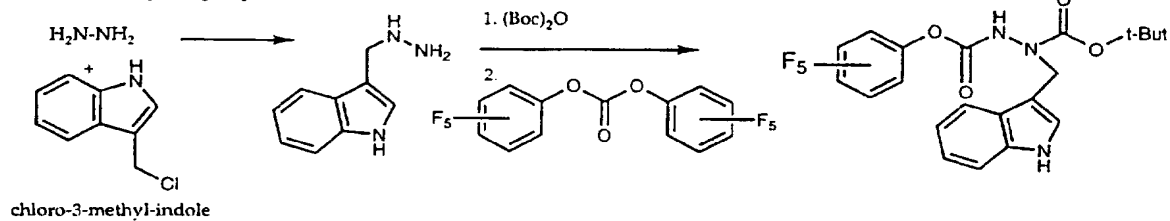


FIGURE 11

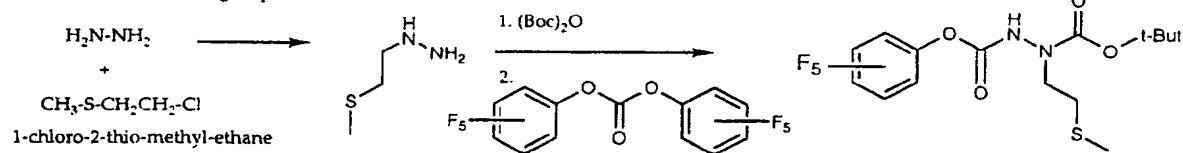
## 6. Azatide with Phe - R- group:



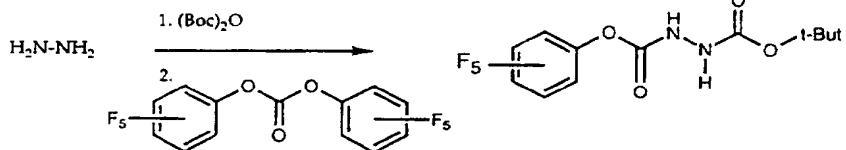
## 7. Azatide with Trp - R- group:



## 8. Azatide with Met - R- group:



## 9. Azatide with Gly - R- group:



## 10. Azatide with Ser - R- group:

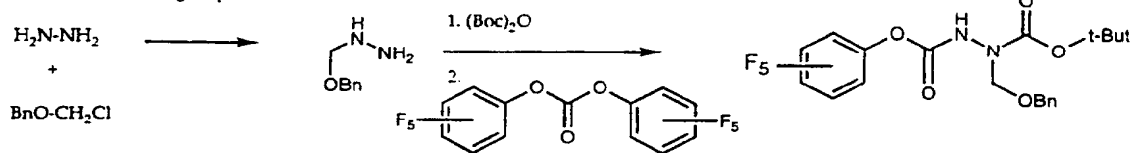


FIGURE 12

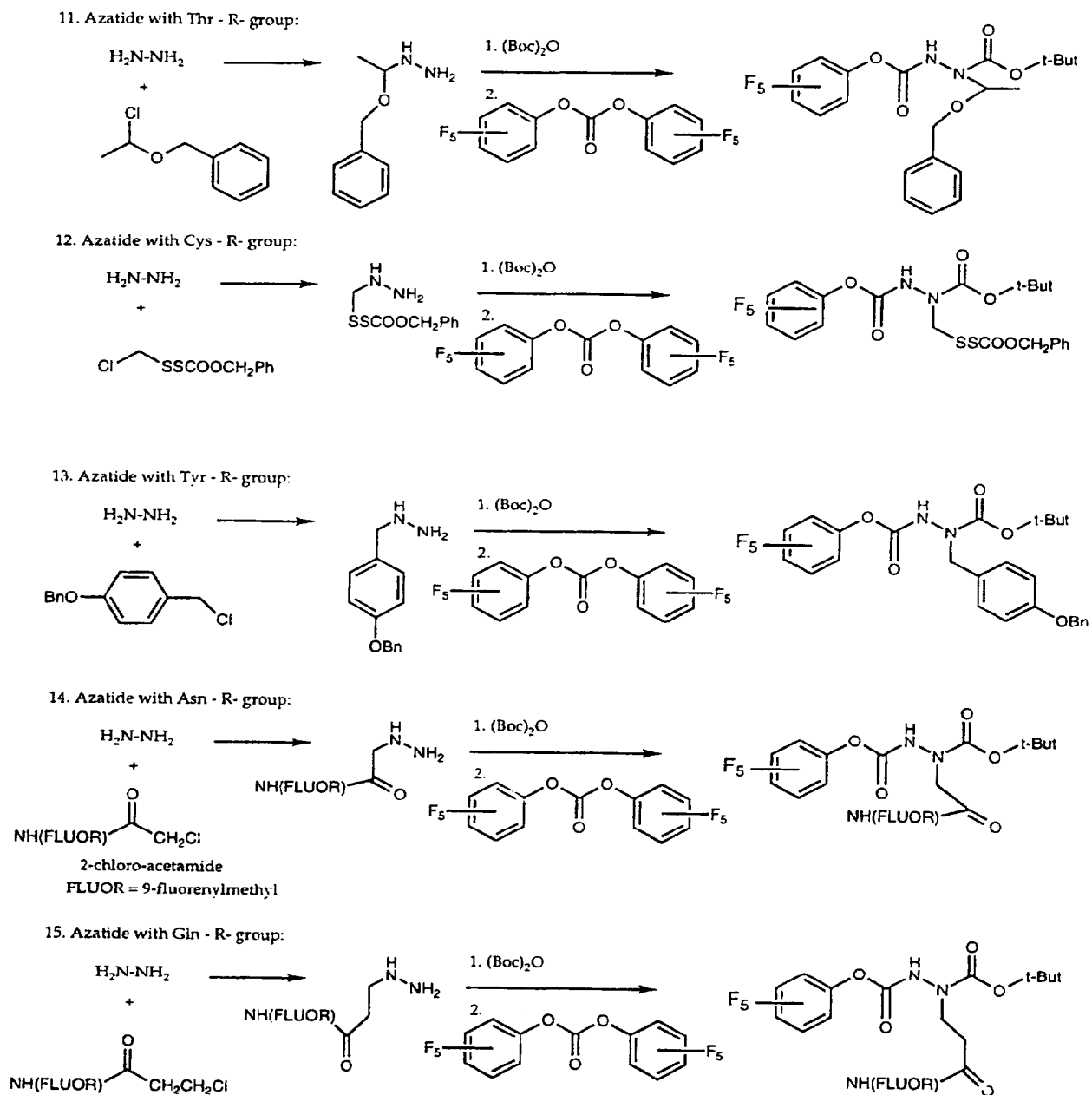
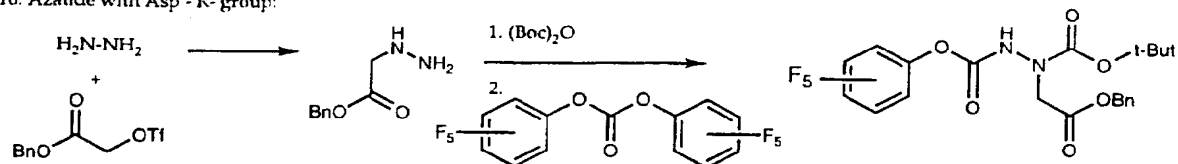
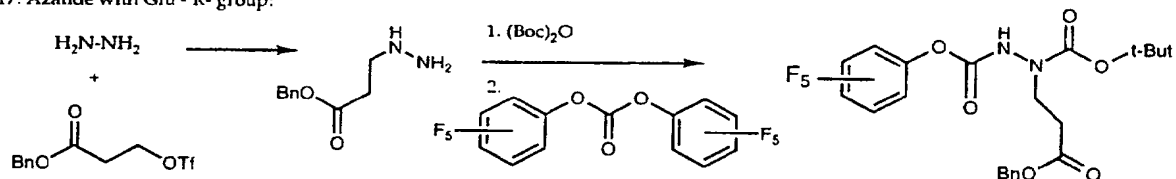


FIGURE 13

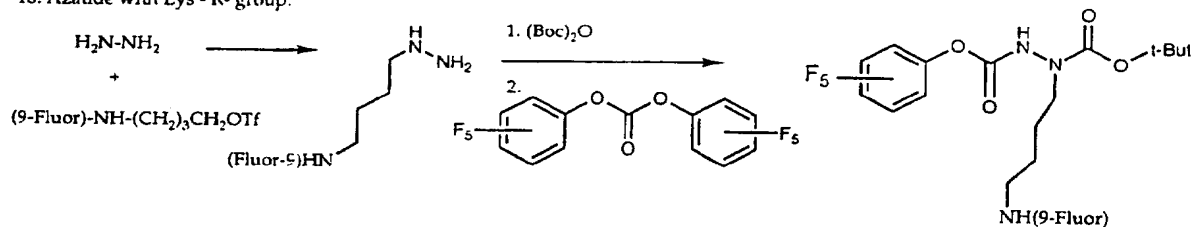
**16. Azatide with Asp - R- group:**



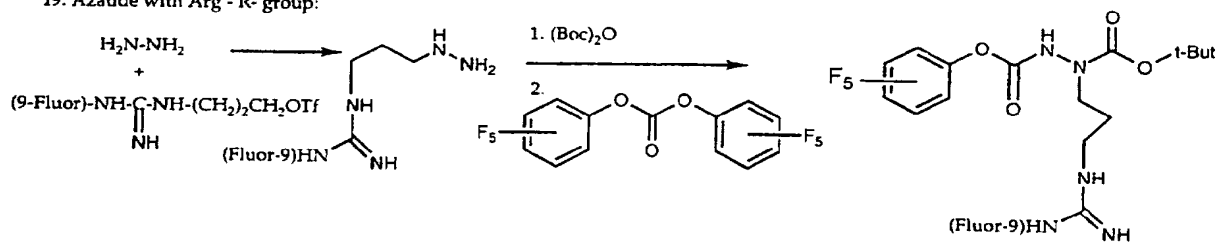
17. Azanide with Glu - R- group:



18. Azatide with Lys - R- group:



19. Azatide with Arg - R- group:



**20. Azatide with His - R- group:**

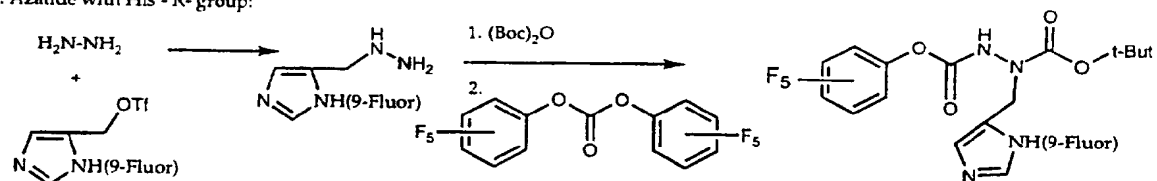
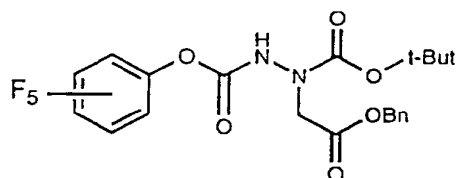
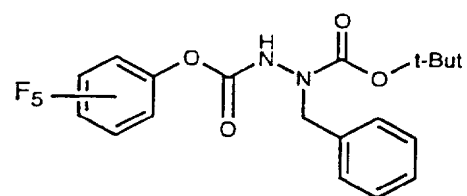


FIGURE 14

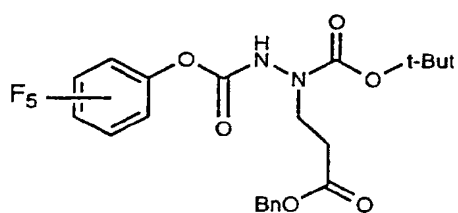
Azatide with Asp - R- group:



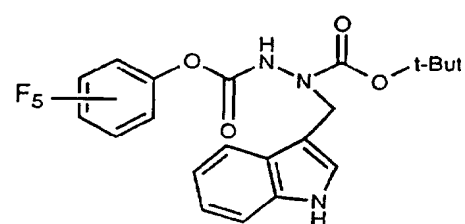
Azatide with Phe - R- group:



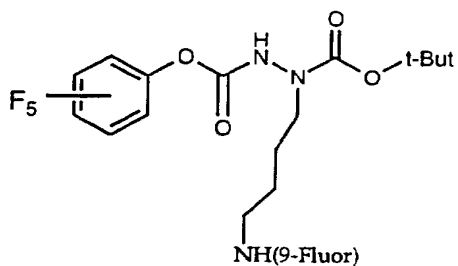
Azatide with Glu - R- group:



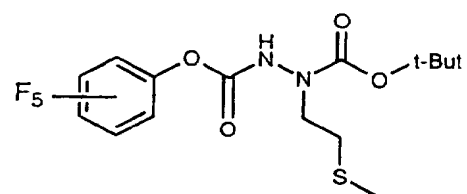
Azatide with Trp - R- group:



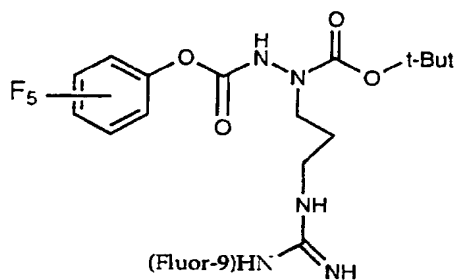
Azatide with Lys - R- group:



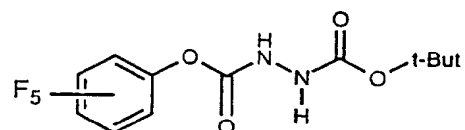
Azatide with Met - R- group:



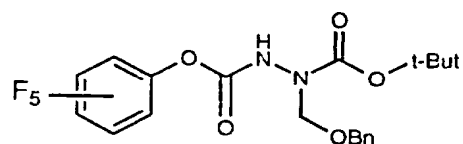
Azatide with Arg - R- group:



Azatide with Gly - R- group:



Azatide with Ser - R- group:



Azatide with His - R- group:

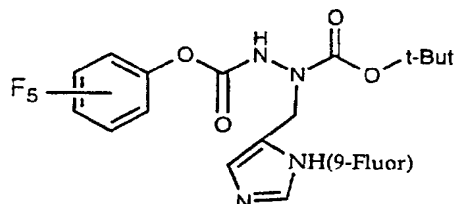
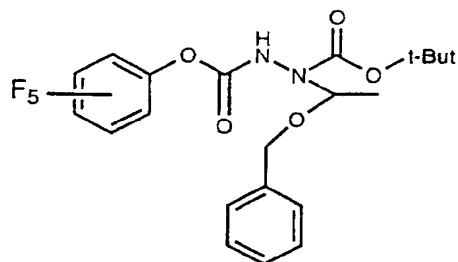
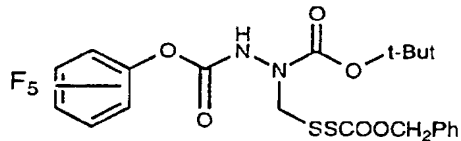


FIGURE 15

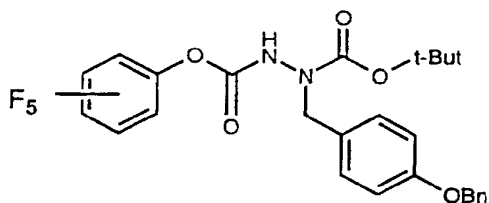
Azatide with Thr - R- group:



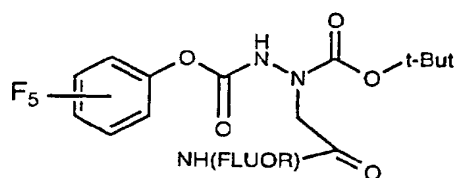
Azatide with Cys - R- group:



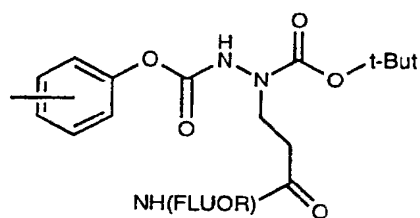
Azatide with Tyr - R- group:



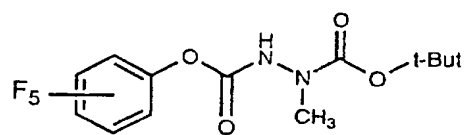
Azatide with Asn - R- group:



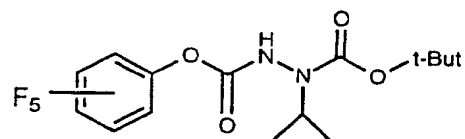
Azatide with Gln - R- group:



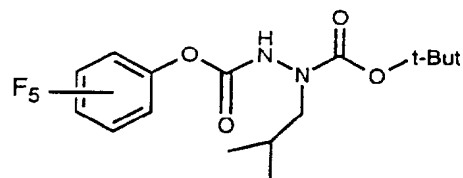
Azatide with Ala - R- group:



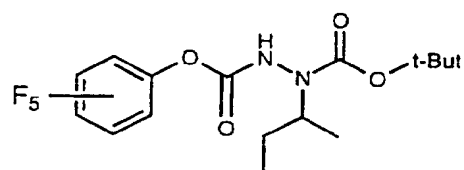
Azatide with Val - R- group:



Azatide with Leu - R- group:



Azatide with Ile - R- group:



Azatide with Pro - R- group:

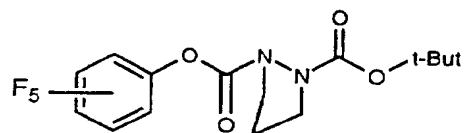


FIGURE 16

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/04963

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/53; C07K 5/02, 1/04

US CL : 435/7.1; 436/518; 530/323, 330, 334, 335

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1; 436/518; 530/323, 330, 334, 335

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS

search terms: azatide, aza peptide, aza amino acid, boc

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAN et al. Azatides: Solution and Liquid Phase Syntnthesis of a New Peptidomimetic. Journal of the American Chemical Society. 20 March 1996. Vol. 118. No. 11. pages 2539-2544, see entire article.	1-10
X --- A	WO 96/03418 (THE SCRIPPS RESEARCH INSTITUTE) 08 February 1996, see Examples 12A and 12B, and Figures 14, 15, 36 and 38.	7-10 ----- 1-6
A	GANTE. Azapeptides. Synthesis. June 1989. Vol. 6. pages 405-413, see entire article.	1-10

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

16 MAY 1997

Date of mailing of the international search report

24 JUN 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

LOKAM GREEN

Telephone No. (703) 308-0196